ENT COOPERATION TREA

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
T3077 (C)/rkk		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/EP 99/ 08323	22/10/1999	27/10/1998
Applicant		
UNILEVER PLC et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

[ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

[X] contained in the international application in written form.

[ ] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[X] furnished subsequently to this Authority in computer readble form.

[X] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[X] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

[ ] the text is approved as submitted by the applicant.

[X] the text has been established by this Authority to read as follows:

ANTIGEN-BINDING PROTEINS COMPRISING A LINKER WHICH CONFERS RESTRICTED CONFORMATIONAL FLEXIBILITY

5. With regard to the **abstract,**

[X] *the text is approved as submitted by the applicant.*

[ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____

[ ] as suggested by the applicant.

[ ] because the applicant failed to suggest a figure.

[ ] because this figure better characterizes the invention.

[X] None of the figures.

PATENT COOPERATION TREATY

REC'D 11 JAN 2001

WIPO PCT

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

15

Applicant's or agent's file reference T3077(C)/rkk	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/EP99/08323	International filing date *(day/month/year)* 22/10/1999	Priority date *(day/month/year)* 27/10/1998
International Patent Classification (IPC) or national classification and IPC C12N15/11		
Applicant UNILEVER PLC et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 9 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☒ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
25/05/2000	08.01.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Page, M Telephone No. +49 89 2399 7322

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP99/08323

I. Basis of the report

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).)*:
Description, pages:

1-25 as originally filed

Claims, No.:

1-14 as originally filed

Drawings, sheets:

1/7-7/7 as originally filed

Sequence listing part of the description, pages:

26-39 (SEQ ID NOs. 1-45), as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☒ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☒ furnished subsequently to this Authority in computer readable form.
- ☒ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☒ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:
   see separate sheet

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	
	No:	Claims	1-14
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-14
Industrial applicability (IA)	Yes:	Claims	1-14
	No:	Claims	

2. Citations and explanations
   see separate sheet

VI. Certain documents cited

1. Certain published documents (Rule 70.10)

and / or

2. Non-written disclosures (Rule 70.9)

   see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:
see separate sheet

VIII. Certain observations on th int rnational applicati n

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

The application concerns the use of short (4-30 amino acid residue) peptide linkers to join binding domains in multivalent binding proteins. Protection is particularly sought for linkers which confer restricted conformational flexibility. Although the application provides specific examples of suitable linkers, the technical features necessary for conferring or assessing restricted conformational flexibility are not clear.

Re Item I

Basis of the report

The examination is being carried out on the **following application documents:**

Text for the Contracting States:
AT BE CH DE DK ES FI FR GB GR IT IE LI LU MC NL PT SE

Description, pages:

1-25 as originally filed

Claims, No.:

1-14 as originally filed

Drawings, sheets:

1/7-7/7 as originally filed

Re Item V

Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1) Reference is made to the following documents:
   D1: WO 93 02198 A (TECHNICAL RESEARCH CENTRE OF FINLAND) 4 February 1993 (1993-02-04)
   D2: H. HOOGENBOOM: 'Mix and match: Building manifold binding sites.' NATURE BIOTECHNOLOGY, vol. 15, no. 2, February 1997 (1997-02), pages 125-126, XP002110046 New York, NY, USA

D3: P. HOLLIGER ET AL.: 'Specific killing of lymphoma cells by cytotoxic T-cells mediated by a bispecific diabody.' PROTEIN ENGINEERING, vol. 9, no. 3, March 1996 (1996-03), pages 299-305, XP002112075 Oxford, GB

D4: K. ALFTHAN ET AL.: 'Properties of a single-chain antibody containing different linker peptides.' PROTEIN ENGINEERING, vol. 8, no. 7, July 1995 (1995-07), pages 725-731, XP002112077 Oxford, GB

D5: WO 94 13804 A (CAMBRIDGE ANTIBODY TECHNOLOGY LTD. & MEDICAL RESEARCH COUNCIL) 23 June 1994 (1994-06-23)

D6: WO 97 14719 A (UNILEVER NV) 24 April 1997 (1997-04-24)

2) **Novelty - Art.33(1) and (2) PCT**:

Claims 1-5 and 8-14 lack novelty in light of D4, which discloses the use of the peptide sequence ATTTGSSPGPT as a linker between the V_H and V_L domains of a single chain antibody (D4 Fig 1). The disclosed antibody is considered to fall within the scope of the claims of the present application as can be understood as being a multivalent binding protein (taking V_H and V_L as separate binding units).

Claims 1-3, 5 and 8-14 also lack novelty in light of D1 and D4, which disclose the use of a number of proline-containing (inflexible) linker regions for joining protein binding domains (D1 page 13 lines 4-8 and page 16 lines 26-28; D4 linkers L0, L1 and L5 in Fig. 1).

Claims 6 and 7 lack novelty in light of D2 and D6, which disclose fusion protein constructs comprising two (camel) V_H domains joined by an oligopeptide linker (D2 Fig. 1 and D6 page 8 lines 24-36).

N.B.: Novelty could haven been restored to the above claims if the subject matter had been restricted to the novel linker sequences of claim 4.

3) **Inventive Step - Art.33(1) and (3) PCT**:

Claims 1-14 have been found to lack novelty and therefore also inventive step.

Note for further examination:

The closest prior art is document D6, which discloses polypeptide constructs comprising two V_H domains joined by an intermediate peptide linker (D6 page 8 lines 24-36).

In the light of the prior art, the technical problem can be regarded as the provision of alternative linker polypeptides for joining protein binding domains (e.g. V_H domains).

The technical problem is solved by the subject matter of claim 4, which provides three alternative linker sequences.

The use of the polypeptide sequence ATTGSSPGPT as a linker between protein binding domains in multivalent proteins has been disclosed (D4 Fig. 1) and can therefore not be regarded as being novel or inventive. However, even if claim 4 were restricted to the SSASASSA and GSPGSPG it cannot be seen how inventive step could be acknowledged. Such linker sequences are already taught by the prior art and the specific sequences are easily derived therefrom (e.g. those provided by D4 Fig. 1; D1 Fig. 1; D4 page 299 right-hand column paragraph 2). In fact, according to Fig. 5 of the present application, the sequence ATTGSSPGPT from D4 provides better binding capacity than either of the other two sequences.

Re Item VI
Certain documents cited

Certain published documents (Rule 70.10)

Application No Patent No	Publication date *(day/month/year)*	Filing date *(day/month/year)*	Priority date *(valid claim)* *(day/month/year)*
99303118.6	24/05/00	27/10/98	27/10/98
EP98/06991	14/05/99	27/10/98	27/10/97

Re Item VII
Certain defects in the international application

a) Contrary to the requirements of Rule 5.1(a)(ii) PCT, the relevant background art disclosed in the documents D1-D6 are not mentioned in the description, nor are these documents identified therein.

b) The terms "100:g" on line 14 of page 11 and "stuffer sequence" on line 19 of page 15 lack clarity (Article 5 PCT).

The overall clarity and conciseness of the description are appreciated by the examiner.

Re Item VIII
Certain observations on the international application

a) The term "confers restricted conformational flexibility" in claims 1, 5, 12 and dependent claims is unclear and renders the scope of the claims obscure. The term is relative, having no standardised meaning in the art and not being adequately defined in the description (the statement on page 4 that movement of the antigen binding units about the backbone of the linker is not consideredto be an adequate description). For the purpose of examination, the term has been understood as applying to any polypeptide linker, since taken literally all peptide bonds are restricted in their flexibility.

b) Naturally occurring polyvalent binding proteins (e.g. antibodies) fall within the scope of the claims of the present application, as the flexible hinge region of immunoglobulins contains proline residues (D6 page 727 right-hand column paragraph 1 and Fig. 1) (Article 6 PCT).

PCT REQUEST

T3077(C)/rkk

Original (for **SUBMISSION**) - printed on 20.10.1999 03:36:25 PM

0 0-1	**For receiving Office use only** International Application No.	PCT/EP 99/08323 09/807172
0-2	International Filing Date	22 OCT 1999 (22.10.1999)
0-3	Name of receiving Office and "PCT International Application"	EUROPEAN PATENT OFFICE PCT INTERNATIONAL APPLICATION
0-4 0-4-1	**Form - PCT/RO/101 PCT Request** Prepared using	PCT-EASY Version 2.84 (updated 01.01.1999)
0-5	**Petition** The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty	
0-6	**Receiving Office (specified by the applicant)**	European Patent Office (EPO) (RO/EP)
0-7	**Applicant's or agent's file reference**	T3077(C)/rkk
I	**Title of invention**	ANTIGEN-BINDING PROTEINS
II	**Applicant**	
II-1	This person is:	applicant only
II-2	Applicant for	AP: (GH GM KE LS MW SD SL SZ UG ZW); EP: (CY GB IE); AE AU BB CA GB GD GH GM IL KE LC LK LS MN MW NZ SD SG SL TT TZ UG ZA ZW
II-4	Name	UNILEVER PLC
II-5	Address:	Unilever House Blackfriars London, EC4P 4BQ United Kingdom
II-6	State of nationality	GB
II-7	State of residence	GB
II-8	Telephone No.	(01234) 22 2068
II-9	Facsimile No.	(01234) 22 2633

PCT REQUEST

T3077(C)/rkk

Original (for **SUBMISSION**) - printed on 20.10.1999 03:36:25 PM

III-1	**Applicant and/or inventor**	
III-1-1	This person is:	applicant only
III-1-2	Applicant for	EA: (AM AZ BY KG KZ MD RU TJ TM); EP: (AT BE CH&LI DE DK ES FI FR GR IT LU MC NL PT SE); OA: (BF BJ CF CG CI CM GA GN GW ML MR NE SN TD TG); AL AM AT AZ BA BG BR BY CH&LI CN CR CU CZ DE DK DM EE ES FI GE HR HU ID IS JP KG KP KR KZ LR LT LU LV MD MG MK MX NO PL PT RO RU SE SI SK TJ TM TR UA UZ VN YU
III-1-4	Name	UNILEVER NV
III-1-5	Address:	Weena 455 NL-3013 AL Rotterdam Netherlands
III-1-6	State of nationality	NL
III-1-7	State of residence	NL
III-2	**Applicant and/or inventor**	
III-2-1	This person is:	applicant only
III-2-2	Applicant for	IN
III-2-4	Name	HINDUSTAN LEVER LIMITED
III-2-5	Address:	Hindustan Lever House 165/166 Backbay Reclamation Maharashtra 400 020 Mumbai India
III-2-6	State of nationality	IN
III-2-7	State of residence	IN
III-3	**Applicant and/or inventor**	
III-3-1	This person is:	applicant and inventor
III-3-2	Applicant for	US only
III-3-4	Name (LAST, First)	FRENKEN, Leon, Gerardus, Joseph
III-3-5	Address:	Unilever Research Colworth Colworth House Sharnbrook Bedford, Bedfordshire MK44 1LQ United Kingdom
III-3-6	State of nationality	NL
III-3-7	State of residence	GB
III-4	**Applicant and/or inventor**	
III-4-1	This person is:	applicant and inventor
III-4-2	Applicant for	US only
III-4-4	Name (LAST, First)	HOWELL, Steven
III-4-5	Address:	Unilever Research Colworth Colworth House Sharnbrook Bedford, Bedfordshire MK44 1LQ United Kingdom
III-4-6	State of nationality	GB
III-4-7	State of residence	GB

PCT REQUEST

T3077(C)/rkk

Original (for **SUBMISSION**) - printed on 20.10.1999 03:36:25 PM

III-5	**Applicant and/or inv ntor**	
III-5-1	This person is:	applicant and inventor
III-5-2	Applicant for	US only
III-5-4	Name (LAST, First)	VAN DER VAART, Jan, Marcel
III-5-5	Address:	Unilever Research Vlaardingen Olivier van Noortlaan 120 NL-3133 AT Vlaardingen Netherlands
III-5-6	State of nationality	NL
III-5-7	State of residence	NL
IV-1	**Agent or common representative; or address for correspondence** The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as:	agent
IV-1-1	Name (LAST, First)	EVANS, Jacqueline, Gail, Victoria
IV-1-2	Address:	Unilever PLC, Patent Department Colworth House Sharnbrook Bedford, Bedfordshire MK44 1LQ United Kingdom
IV-1-3	Telephone No.	(01234) 22 2644
IV-1-4	Facsimile No.	(01234) 22 2633
V	**Designation of States**	
V-1	Regional Patent (other kinds of protection or treatment, if any, are specified between parentheses after the designation(s) concerned)	AP: GH GM KE LS MW SD SL SZ UG ZW and any other State which is a Contracting State of the Harare Protocol and of the PCT EA: AM AZ BY KG KZ MD RU TJ TM and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT EP: AT BE CH&LI CY DE DK ES FI FR GB GR IE IT LU MC NL PT SE and any other State which is a Contracting State of the European Patent Convention and of the PCT OA: BF BJ CF CG CI CM GA GN GW ML MR NE SN TD TG and any other State which is a member State of OAPI and a Contracting State of the PCT
V-2	National Patent (other kinds of protection or treatment, if any, are specified between parentheses after the designation(s) concerned)	AE AL AM AT AU AZ BA BB BG BR BY CA CH&LI CN CR CU CZ DE DK DM EE ES FI GB GD GE GH GM HR HU ID IL IN IS JP KE KG KP KR KZ LC LK LR LS LT LU LV MD MG MK MN MW MX NO NZ PL PT RO RU SD SE SG SI SK SL TJ TM TR TT TZ UA UG US UZ VN YU ZA ZW

PCT REQUEST

T3077(C)/rkk

Original (for **SUBMISSION**) - printed on 20.10.1999 03:36:25 PM

V-5	**Precautionary Designati n Statement** In addition to the designations made under items V-1, V-2 and V-3, the applicant also makes under Rule 4.9(b) all designations which would be permitted under the PCT except any designation(s) of the State(s) indicated under item V-6 below. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit.		
V-6	**Exclusion(s) from precautionary designations**	NONE	
VI-1	**Priority claim of earlier international application**		
VI-1-1	Filing date	27 October 1998 (27.10.1998)	
VI-1-2	Number	PCT/EP98/06991	
VI-1-3	PCT receiving Office	EP	
VI-2	**Priority claim of earlier regional application**		
VI-2-1	Filing date	22 April 1999 (22.04.1999)	
VI-2-2	Number	99303118.6	
VI-2-3	Regional Office	EP	
VI-3	**Priority document request** The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) identified above as item(s):	VI-1, VI-2	
VII-1	**International Searching Authority Chosen**	European Patent Office (EPO) (ISA/EP)	
VIII	**Check list**	number of sheets	electronic file(s) attached
VIII-1	Request	5	-
VIII-2	Description (excluding sequence listing part)	25	-
VIII-3	Claims	2	-
VIII-4	Abstract	1	t3077.txt
VIII-5	Drawings	7	-
VIII-6	Sequence listing part of description	14	-
VIII-7	TOTAL	54	
	Accompanying items	paper document(s) attached	electronic file(s) attached
VIII-8	Fee calculation sheet	✓	-
VIII-10	Copy of general power of attorney	reference no. 170	-
VIII-15	Nucleotide and/or amino acid sequence listing in computer readable form		separate diskette
VIII-16	PCT-EASY diskette	-	diskette
VIII-18	**Figure of the drawings which should accompany the abstract**		
VIII-19	**Language of filing of the international application**	English	
IX-1	**Signature of applicant r agent**		
IX-1-1	Nam (LAST, First)	EVANS, Jacqueline, Gail, Victoria	

PCT REQUEST

T3077(C)/rkk

Original (for **SUBMISSION**) - printed on 20.10.1999 03:36:25 PM

FOR RECEIVING OFFICE USE ONLY

10-1	Dat of actual receipt f the purp rted international application	22 OCT 1999 (22.10.1999)
10-2	Drawings:	
10-2-1	Received	X
10-2-2	Not received	
10-3	Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application	
10-4	Date of timely receipt of the required corrections under PCT Article 11(2)	
10-5	International Searching Authority	ISA/EP
10-6	Transmittal of search copy delayed until search fee is paid	

FOR INTERNATIONAL BUREAU USE ONLY

11-1	Date of receipt of the record copy by the International Bureau	

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 29 June 2000 (29.06.00)	
International application No. PCT/EP99/08323	**Applicant's or agent's file reference** T3077(C)/rkk
International filing date (day/month/year) 22 October 1999 (22.10.99)	**Priority date** (day/month/year) 27 October 1998 (27.10.98)
Applicant FRENKEN, Leon, Gerardus, Joseph et al	

1. The designated Office is hereby notified of its election made:

   [X] in the demand filed with the International Preliminary Examining Authority on:

   25 May 2000 (25.05.00)

   [ ] in a notice effecting later election filed with the International Bureau on:

   _____

2. The election [X] was

   [ ] was not

   made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer

Manu Berrod

Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

KAN, Jacob, Hendrik
Unilever NV
Patent Department
P.O. Box 137
NL-3130 AC Vlaardingen
PAYS-BAS

Date of mailing (day/month/year)
08 December 2000 (08.12.00)

Applicant's or agent's file reference
T3077(V)

IMPORTANT NOTIFICATION

International application No.
PCT/EP99/08323

International filing date (day/month/year)
22 October 1999 (22.10.99)

1. The following indications appeared on record concerning:

[ ] the applicant [ ] the inventor [X] the agent [ ] the common representative

Name and Address

EVANS, Jacqueline, Gail, Victoria
Unilever PLC, Patent Dept.
Colworth House
Sharnbrook
Bedford, Bedfordshire MK44 1LQ
United Kingdom

State of Nationality:
State of Residence:
Telephone No.: (01234) 22 2644
Facsimile No.: (01234) 22 2633
Teleprinter No.:

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[X] the person [X] the name [X] the address [ ] the nationality [X] the residence

Name and Address

KAN, Jacob, Hendrik
Unilever NV
Patent Department
P.O. Box 137
NL-3130 AC Vlaardingen
Netherlands

State of Nationality:
State of Residence:
Telephone No.:
Facsimile No.:
Teleprinter No.:

3. Further observations, if necessary:
Please also note the new agent's reference number.

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[X] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer
A. Karkachi

Telephone No.: (41-22) 338.83.38

Form PCT/IB/306 (March 1994)

003713336

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ETATS-UNIS D'AMERIQUE

In its capacity as designated Office

NOTIFICATION CONCERNING DOCUMENT TRANSMITTED

Date of mailing (day/month/year)	
02 May 2000 (02.05.00)	
International application No.	**International filing date (day/month/year)**
PCT/EP99/08323	22 October 1999 (22.10.99)
Applicant	
UNILEVER PLC et al	

The International Bureau transmits herewith the following documents and number thereof:

_____ cop(ies) of priority document(s) (Rule 17.2(a))

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer

Marie-José Devillard

Telephone No.: (41-22) 338.83.38

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 99/08323

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/11 C07K19/00 C07K16/46 C12N1/19 C12N15/81

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 93 02198 A (TECHNICAL RESEARCH CENTRE OF FINLAND) 4 February 1993 (1993-02-04) page 11 -page 20 figures 2,4 claims	1-5,8-14
Y	---	6,7
Y	H. HOOGENBOOM: "Mix and match: Building manifold binding sites." NATURE BIOTECHNOLOGY, vol. 15, no. 2, February 1997 (1997-02), pages 125-126, XP002110046 New York, NY, USA page 126, right-hand column, line 30 - line 39 figure 1	6,7

	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 99/08323

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(54) Title: ANTIGEN-BINDING PROTEINS

(57) Abstract

Use of a polypeptide linker group, the amino acid sequence of which group confers restricted conformational flexibility, as a linking group to link binding units, preferably antigen binding units comprising heavy chain variable domains derived from an immunoglobulin naturally devoid of light chains, in a multivalent binding protein.

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ANTIGEN-BINDING PROTEINS

FIELD OF THE INVENTION

The present invention relates to the preparation of multivalent and multispecific binding proteins. In particular, the invention relates to the preparation of antigen binding proteins comprising a plurality of binding units linked in series by means of intervening polypeptide linker groups, the amino acid sequence of which linker groups confer restricted conformational flexibility.

BACKGROUND OF THE INVENTION

There is considerable interest in the preparation of multivalent and/or multispecific antigen binding proteins. Antigen binding proteins which are multivalent (that is, comprise more than one antigen binding site), more especially those which are also multispecific (where the antigen binding sites have differing antigen specificities) have found particular application in the fields of diagnosis or therapy, for example, where the construction of binding proteins having binding activity against both target site and diagnostic or therapeutic agent allows for targeted delivery of the diagnostic or therapeutic agent to the intended site of action. Other uses for which multivalent and multispecific binding proteins have been proposed include assays, such as immunoassays and agglutination assays, and purification processes.

Those multivalent, multispecific antigen binding proteins which have been described in the literature to date rely, in general, on the association of antibody light and heavy chain variable domains for the formation of the antigen binding site.

Thus, constructs comprising two or more polypeptide chains are described in WO 94/09131 (Scotgen Limited) and WO 97/14719

(Unilever) and WO 97/38102 (Unilever); multivalent molecules comprising two or more single chain Fv molecules linked together are described in WO 93/11161 (Enzon Inc.) and WO 94/13806 (Dow Chemical Co.).

WO 94/04678 (Casterman et al) describes immunoglobulins capable of exhibiting the functional properties of classical, four chain, immunoglobulins but which comprise two heavy polypeptide chains only and are naturally devoid of light polypeptide chains. Fragments corresponding to isolated VH domains or to VH dimers linked by the hinge disulphide are also disclosed. These immunoglobulins, which may be isolated from Camelids, do not rely on the association of heavy and light chain variable domains for the formation of the antigen-binding site; instead, the heavy chain variable domain (hereinafter VHH) alone forms the complete antigen binding site, constituting a single domain binding site.

In their later patent application, WO 96/34103, Casterman et al disclose multivalent, multispecific constructs comprising VHH fragments combined with a linker sequence. Suitable linker sequences disclosed and exemplified are derived from sequences corresponding to the hinge domain of an immunoglobulin devoid of light chains.

In the Applicant's co-pending patent publication number WO 99/23221, published after the priority date of the present application, there are disclosed multivalent, multispecific antigen-binding proteins comprising a polypeptide comprising in series two or more single domain binding units which are preferably variable domains of a heavy chain derived from an immunoglobulin naturally devoid of light chains. The individual single domain binding units may suitably be linked by means of peptide linkers, preferably flexible peptide linkers, which allow the variable domains to flex in relation to each other

with the aim of ensuring that they can bind to multiple antigenic determinants simultaneously.

There remains a continuing need for the development of improved methods for producing multivalent and/or multispecific binding proteins, especially antigen binding proteins. In particular, there is commercial interest in producing molecules which not only have improved binding activity but which also can be produced economically on a large scale.

SUMMARY OF THE INVENTION

In a first aspect, the invention provides the use of a polypeptide group, the amino acid sequence of which group confers restricted conformational flexibility, as a linking group to link binding units in a multivalent binding protein.

The invention also provides a multivalent binding protein comprising a plurality of binding units linked by means of intervening polypeptide linker groups, the amino acid sequence of which linker group confers restricted conformational flexibility.

The invention further provides a nucleotide sequence encoding a multivalent antigen binding protein according to the invention and cloning and expression vectors comprising such nucleotide sequences. Also provided are host cells transformed with vectors comprising such nucleotide sequences.

As used herein, a 'multivalent binding protein' is a protein which has more than one binding units which allow for specific binding with a molecule partner in a binding pair. Included within this are bivalent, trivalent and so on. Examples of suitable binding units include antigen binding domains of antibodies, binding domains of receptors such as hormone receptors, lectins, enzymes, and cell adhesion molecules. A

'multivalent antigen binding protein' is a protein which has more than one antigen binding unit.

An 'antigen binding unit' is any structure which exhibits antigen-binding activity. This may be an antibody or an immunologically active fragment thereof. An 'antibody' refers to an immunoglobulin which may be derived from natural sources or synthetically produced. Unless indicated otherwise, 'antibody' and 'immunoglobulin' are used synonymously throughout this specification.

An antibody fragment is a portion of a whole antibody which retains the ability to exhibit antigen-binding activity. The antigen binding site may be formed through association of antibody light and heavy chain variable domains or may comprise individual antibody variable domains, constituting a single domain binding site.

Suitable fragments include Fab (comprising an antibody light chain associated with the V_H and C_{H1} domains of an antibody heavy chain), Fv (comprising the variable domains of antibody heavy and light chains associated with each other) and scFv (comprising an antibody V_H domain linked to a V_L domain by a flexible peptide linker) fragments. Where the antigen binding site comprises a single variable domain, this may be a heavy chain variable domain, most suitably a heavy chain variable domain derived from an immunoglobulin naturally devoid of light chains.

'Restricted conformational flexibility' relates to restriction of movement of the antigen binding units about the backbone of the intervening polypeptide linker group.

The present invention may be more fully understood with reference to the following description, when read together with the accompanying drawings. For convenience, an antigen binding

protein comprising two single binding units is hereinafter referred to as a 'bi-head'.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a nucleotide sequence of the PstI-BstEII insert of plasmid pUR4640, encoding the heavy chain variable domain of an anti-RR6 antibody (denoted R9) from a llama.

Figure 2 shows the nucleotide sequence of the PstI-BstEII insert of plasmid pUR4601, encoding the heavy chain variable domain of an anti-hCG antibody (denoted H14) from a llama.

Figure 3 shows a map of plasmid pUR4619.

Figure 4 shows the nucleotide sequence within plasmid pUR4619 which encodes an anti-hCG-anti-RR6 bispecific biheaded antigen-binding protein (denoted HI4-R9), missing the first 4 and last 3 amino acids.

Figure 5 shows the A405 signals of an ELISA to determine bispecificity of various HI4-R9 biheads.

Figure 6 shows the scores achieved in a rapid assay technology (RAT) format assay following the detection of 1 IU/ml hCG (human chorionic gonadotrophin protein) with various anti-hCG-anti-RR6 bihead antigen binding proteins derived from a llama wherein the anti-hCG and anti-RR6 fragments are linked as follows (see Example 1.5):

Number	Linker	
1	no linker (directly attached)	
2	G-T-S-G-S	(SEQ. ID NO. 1)
3	S-S-S-A-S-A-S-S-A	(SEQ. ID NO. 2)
4	G-S-P-G-S-P-G	(SEQ. ID NO. 3)
5	A-T-T-T-G-S-S-P-G-P-T	(SEQ. ID NO. 4)
6	A-N-H-S-G-N-A-S	(SEQ. ID NO. 5)

Figure 7 shows a comparison of the sensitivity of detection of hCG in a RAT assay using various biheads (see Example 1.5).

DETAILED DESCRIPTION OF THE INVENTION

The invention is based on the unexpected finding that by using a polypeptide linking group conferring restricted conformational flexibility to link together antigen binding units, multivalent antigen binding proteins having advantageous binding affinity, as demonstrated by their increased sensitivity of diagnosis and detection, are obtained. Furthermore, constructs according to the invention may conveniently be produced at high yields economically and efficiently on a scale appropriate for industrial use.

As is apparent from the discussion of the background to the invention above, to the extent that multivalent antigen binding constructs comprising separate binding units linked together have been described at all in the literature, the linking means has been provided by flexible peptide groups. Flexibility of conformation in the linker group has been considered desirable in order to allow the multivalent construct to assume the correct orientation to allow simultaneous binding of multiple antigens.

Surprisingly, the present inventors have found that by restricting the conformational flexibility of the linking polypeptide group, multivalent antigen binding constructs having improved binding affinity may be obtained. This is entirely contrary to the teaching in the art that the linking group should desirably be flexible.

The invention is applicable to the preparation of multivalent antigen binding constructs comprising antigen binding units where the antigen binding site is formed through association of antibody light and heavy chain variable domains. Preferably, however, the constructs prepared according to the invention comprise a plurality of single domain binding units, more particularly a plurality of heavy chain variable domains derived from an immunoglobulin naturally devoid of light chains such as may be obtained from lymphoid cells, especially peripheral blood lymphocytes, bone marrow cells or spleen cells derived from Camelids as described in WO 94/04678 (Casterman et al) discussed above. An advantage of using single domain binding units which are heavy chain variable domains derived from Camelids is that they can readily and conveniently be produced economically on a large scale, for example, using a transformed lower eukaryotic host, as described in WO 94/25591 (Unilever), described above.

It will be appreciated that heavy chain variable domains derived from other immunoglobulins modified ('camelised') to enable them to function as single binding domains in the same way as the heavy chain variable domains derived from Camelids may also suitably be used according to the invention.

Bivalent forms, that is having two antigen binding sites, of the multivalent antigen binding proteins prepared according to the invention are preferred but it will be appreciated that higher multivalent forms, which are also encompassed in the present invention, may find application under suitable circumstances, for example where more than two antigens are required to bind,

for example in processes for scavenging molecules from solution or processes where close proximity of molecules form the basis of an assay.

Structural features which may suitably be incorporated into the linking polypeptide group in order to achieve the effect of restricting conformational flexibility according to the purposes of the invention would readily suggest themselves to those skilled in the art.

Accordingly, in one embodiment, the linker group preferably comprises one or more proline residues.

Without wishing to be bound by theory, it is generally thought that the presence of a proline residue in a peptide sequence encourages the amino acid backbone of the peptide to adopt a beta-turn structural configuration, with the peptide backbone changing direction about the proline residue. Linker groups comprising other sequence features which promote the formation of a beta-turn configuration in the peptide backbone, such as peptide linkers containing valine residues or constrained residues such as 8-bicyclic and 5,9-bicyclic tripeptide units (see, for example, Johannesson et al, J. Med. Chem., 42, 601-608 (1999), may also suitably find application in the present invention.

In another embodiment, peptide linker groups derived from naturally occurring proteins such as cell wall proteins (CWP), in particular, CWP1, or cellobishydrolases (CBH), such as CBH1P, which serve to restrict conformational flexibility or linker groups showing at least 50% homology thereto as determined by the ALIGN program of Dayhoff et al (1983), Methods Enzymol., 91, 524-545, may also suitably be used according to the invention.

Peptide linker groups which encode a glycosylation binding site and/or are resistant to proteolytic attack may also suitably be

employed. Here, the presence of a carbohydrate attached to the amino acid residues has the effect of restricting the flexibility of the peptide backbone.

Conveniently, the polypeptide linking group according to the invention comprises from 4 to 30 amino acid residues, preferably from 5 to 15 amino acid residues.

Preferred polypeptide linking groups according to the invention comprise an amino acid sequence selected from:

S-S-S-A-S-A-S-S-A,	(SEQ. ID NO. 2)
G-S-P-G-S-P-G,	(SEQ. ID NO. 3)
A-T-T-T-G-S-S-P-G-P-T	(SEQ. ID NO. 4)

It will be appreciated that although the invention has been described primarily by reference to antigen binding proteins, it is equally applicable to proteins comprising other binding units as described above. References to antigen binding proteins will accordingly be understood to refer also to such other proteins unless the context dictates otherwise.

Multivalent antigen binding proteins according to the invention may be prepared by transforming a host by incorporating a gene encoding the polypeptide as set forth above and expressing said gene in said host.

Suitably the host or hosts may be selected from prokaryotic bacteria, such as Gram-negative bacteria, for example *E. coli*, and Gram-positive bacteria, for example *B. subtilis* or lactic acid bacteria, lower eukaryotes such as yeasts, for example belonging to the genera *Saccharomyces*, *Kluyveromyces*, *Hansenula* or *Pichia*, or moulds such as those belonging to the genera *Aspergillus* or *Trichoderma*.

Preferred hosts for use in connection with the present invention are the lower eukaryotic moulds and yeasts.

Techniques for synthesising genes, incorporating them into hosts and expressing genes in hosts are well known in the art and the skilled person would readily be able to put the invention into effect using common general knowledge.

Methods for producing antibody fragments or functionalised fragments thereof derived from the heavy chain immunoglobulin of *Camelidae* using a transformed lower eukaryotic host are described, for example in patent application WO 94/25591 and such techniques may suitably be applied to prepare constructs according to the present invention.

Proteins according to the invention may be recovered and purified using conventional techniques such as affinity chromatography, ion exchange chromatography or gel filtration chromatography.

The activity of the multivalent binding proteins according to the invention may conveniently be measured by standard techniques known in the art such as enzyme-linked immunoadsorbant assay (ELISA), radioimmune assay (RIA) or by using biosensors.

The following examples are produced by way of illustration only. Techniques used for the manipulation and analysis of nucleic acid materials were performed as described in Sambrook et al, Molecular Cloning, Cold Spring Harbor Press, New York, 2nd Ed., (1989) unless otherwise indicated.

Restriction sites are underlined.
HC-V denotes heavy chain variable domain.

EXAMPLES

Example 1 Self Assembling Llama Bi-heads Containing Linker Peptides on Latex to Assay hCG

1.1 Construction of Llama Bi-heads with Various Linkers

a) Induction of humeral immune responses in llama

Male llamas were immunised with a water in oil emulsion (1:9 V/V, antigen in water: Specol (Bokhout et al, Vet. Immunol. Immunopath., **2**:, 491-500 (1981)) subcutaneously and intramuscularly. Per immunisation site 0.75-1.5 ml water in oil emulsion was inoculated containing 100:g antigen. The antigens used were: hCG (Sigma), azo-dye RR6 (ICI) which was coupled to BSA via its reactive triazine group. Immunisations were performed according to the following time table: The second immunisation was performed three weeks after the first. The third was performed two weeks after the second immunisation. The immune response was followed by antigen specific ELISAs.

The anti-RR-6 response was measured by using Nunc Covalink plates, which where coated with the azo-dye. After incubation with (diluted) serum samples, the bound llama antibodies were detected via a incubation with poly-clonal rabbit-anti-llama antiserum (obtained via immunising rabbits with llama immunoglobulins which were purified via ProtA and ProtG columns; ID-DLO), followed by an incubation with swine-anti-rabbit immunoglobulins (Dako) conjugated with alkaline phosphatase. Finally the alkaline phosphatase enzyme-activity was determined after incubation with p-nitro-phenyl phosphate and the optical density was measured at 405nm. The anti-hCG response, was measured in essentially the same way using Nunc maxi-sorb plates coated with hCG.

b) Cloning, expressing and screening of llama HC-V fragments

i) Isolation of gene fragments encoding llama HC-V domains

From an immunised llama a blood sample of about 200ml was taken and an enriched lymphocyte population was obtained via Ficoll (Pharmacia) discontinuous gradient centrifugation. From these cells, total RNA was isolated by acid guanidium thiocyanate extraction (e.g. via the method described by Chomczynnski and Sacchi, Analytical Biochemistry, 162: 156-159 (1987). After first strand cDNA synthesis (e.g. with the Amersham first strand cDNA kit), DNA fragments encoding HC-V fragments and part of the long or short hinge region were amplified by PCR using specific primers:

*Pst*I

V_H - 2B 5'-AGGTSMARCTGCAGSAGTCWGG-3' (SEQ. ID NO. 6)

S = C and G, M = A and C, R = A and G, W = A and T,

*Hin*dIII

Lam-07 5'-AACAGTTAAGCTTCCGCTTGCGGCCGCGGAGCTGGGGTCTTCGCTGTGGTGCG-3'
(short hinge) (SEQ. ID NO. 7)

*Hin*dIII

Lam-08 5'-AACAGTTAAGCTTCCGCTTGCGGCCGCTGGTTGTGGTTTTGGTGTCTTGGGTT-3'
(long hinge) (SEQ. ID NO. 8)

Upon digestion of the PCR fragments with *Pst*I (coinciding with codon 4 and 5 of the HC-V domain, encoding the amino acids L-Q) and *Bst*EII (located at the 3'-end of the HC-V gene fragments, coinciding with the amino acid sequence Q-V-T), the DNA fragments with a length between 300 and 400bp (encoding the HC-V domain, but lacking the first three and the last three codons) were purified via gel electrophoresis and isolation from the agarose gel.

ii) Construction of *Saccharomyces cerevisiae* expression plasmids encoding llama HC-V domains

Plasmids pUR4547 and pUR4548 are *Saccharomyces cerevisiae* episomal expression plasmids, derived from pSY1 (Harmsen et al., Gene, 125: 115-123, (1993). From pSY1 the *Pst*I site, located in front of the GAL7 promoter was removed after partial digestion with *Pst*I, incubation with Klenow fragment and subsequent blunt end ligation. After transformation the desired plasmid could be selected on the basis of restriction pattern analysis. Subsequently, the *Bst*EII site in the Leu2 selection marker was removed by replacing the about 410bp *Afl*II/*Pfl*MI fragment with a corresponding fragment in which the *Bst*EII site was removed via a three step PCR mutagenesis, using the primers:

PCR-A:

*Pfl*MI

BOLI 1 5'-GGGAATTCCAATAGGTGGTTAGCAATCG (SEQ. ID NO. 9)

(*Bst*EII)

BOLI 4 5'-GACCAACGTGGTCGCCTGGCAAAACG (SEQ. ID NO. 10)

PCR-B:

(*Bst*EII)

BOLI 3 5'-CGTTTTGCCAGGCGACCACGTTGGTC (SEQ. ID NO. 11)

*Afl*II

BOLI 2 5'-CCCCAAGCTTACATGGTCTTAAGTTGGCGT (SEQ. ID NO. 12)

PCR-A was performed with primers BOLI 1 and BOLI 4 and resulted in an about 130bp fragment with the *Pfl*MI restriction site at the 3'-end and the inactivated *Bst*EII site at the 5'-end. PCR-B was performed with primers BOLI 2 and BOLI 3 and resulted in an about 290bp fragment with the *Afl*II site at the 5'-end. The third PCR was with the fragments obtained from reaction A and B, together with the primers BOLI 1 and BOLI 2.

Finally, the about 1.8kb *Sac*I-*Hin*dIII fragment was replaced with synthetic fragments, having sequences as presented below, resulting the plasmids pUR4547 and pUR4548, respectively.

- *Sac*I/*Hin*dIII fragment of pUR4547

```
  SacI                                                  (SEQ. ID NO. 13-16)
  GAGCTCATCACACAAACAAACAAAACAAAATGATGCTTTTGCAAGCCTTCCCTT
1 ---------+---------+---------+---------+---------+---- 54
  CTCGAGTAGTGTGTTTGTTTGTTTTGTTTTACTACGAAAACGTTCGGAAGGGAA
                                M  M  L  L  Q  A  F  L  F
                                 |→     SUC2 ss

                                                      PstI
  TTCCTTTTGGCTGGTTTTGCAGCCAAAATATCTGCGCAGGTGCAGCTGCAGG
55------+---------+---------+---------+---------+----- 105
  AAGGAAAACCGACCAAAACGTCGGTTTTATAGACGCGTCCACGTCGACGTCC
    L  L  A  G  F  A  A  K  I  S  A  Q  V  Q  L  Q  E
                                          |→

                          BstEII                      HindIII
   AGTCATAATGAGGGACCCAGGTCACCGTCTCCTCATAATGACTTAAGCTT
106----+---------+---------+---------+---------+----- 155
   TCAGTATTACTCCCTGGGTCCAGTGGCAGAGGAGTATTACTGAATTCGAA
    E  S  *  *  G  T  Q  V  T  V  S  S  *  *
      HC-V cassette                ←|
```

and

- *Sac*I/*Hin*dIII fragment of pUR4548

```
  SacI                                                  (SEQ. ID NO. 17-20)
  GAGCTCATCACACAAACAAACAAAACAAAATGATGCTTTTGCAAGCCTTCCTTT
1 ---------+---------+---------+---------+---------+---- 54
  CTCGAGTAGTGTGTTTGTTTGTTTTGTTTTACTACGAAAACGTTCGGAAGGAAA
                                M  M  L  L  Q  A  F  L  F
                                 |→     SUC2 ss

                                                      PstI
  TCCTTTTGGCTGGTTTTGCAGCCAAAATATCTGCGCAGGTGCAGCTGCAGG
55-----+---------+---------+---------+---------+----- 105
  AGGAAAACCGACCAAAACGTCGGTTTTATAGACGCGTCCACGTCGACGTCC
    L  L  A  G  F  A  A  K  I  S  A  Q  V  Q  L  Q  E
                                          |→
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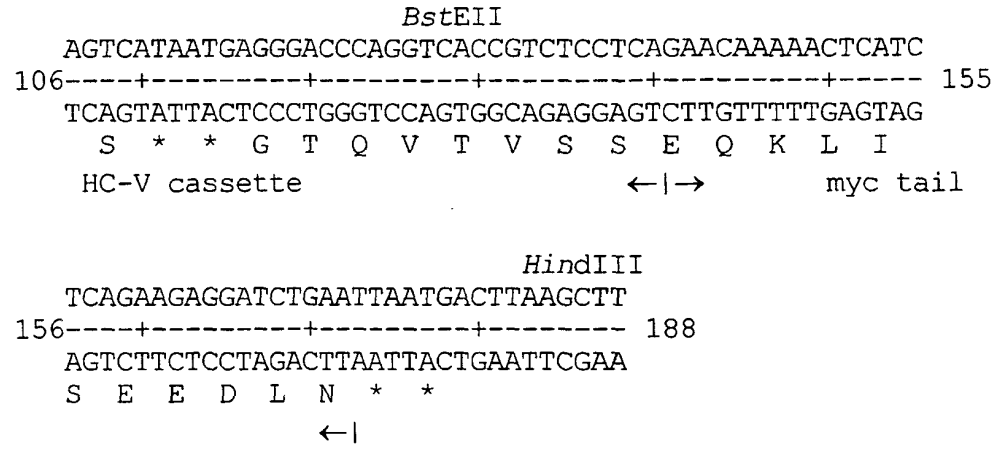


Both plasmids contain the GAL7 promoter and PGK terminator sequences as well as the invertase (SUC2) signal sequence. In both plasmids the DNA sequence encoding the SUC2 signal sequence is followed by the first 5 codons, (encoding Q-V-Q-L-Q) of the HC-V domain (including the *Bst*II site), a stuffer sequence, the last six codons (encoding Q-V-T-V-S-S) of the HC-V domain. In pUR4547, this is followed by two stop codons, an *Afl*II and *Hin*dIII site. In pUR4548, this sequence is followed by eleven codons encoding the myc-tag, two stop codons, an *Afl*II and *Hin*dIII site.

Plasmids pUR4547 and pUR4548 were deposited under the Budapest Treaty at the Centraal Bureau voor Schimmelcultures, Baarn on 18th August 1997 with deposition numbers: CBS 100012 and CBS 100013, respectively. In accordance with Rule 28(4) EPC, or a similar arrangement from a state not being a contracting state of the EPC, it is hereby requested that a sample of such deposit, when requested, will be submitted to an expert only.

Upon digesting pUR4548 with *Pst*I and *Bst*EII, the about 6.4kb vector fragment was isolated and ligated with the *Pst*I-*Bst*EII fragments of about 350bp obtained as described above. After transformation of *S. cerevisiae*, via electroporation, transformants were selected from minimal medium agar plates

(comprising 0.7% yeast nitrogen base, 2% glucose and 2% agar, supplemented with the essential amino acids and bases).

iii) Screening for antigen specific HC-V domains

For the production of llama HC-V fragments with myc-tail, individual transformants were grown overnight in selective minimal medium (comprising 0.7% yeast nitrogen base, 2% glucose, supplemented with the essential amino acids and bases) and subsequently diluted ten times in YPGal medium (comprising 1% yeast extract, 2% bacto pepton and 5% galactose). After 24 and 48 hours of growth, the culture supernatant of the colonies was analysed by ELISA for the presence of HC-V fragments which specifically bind to the antigens hCG, RR6 in essential the same way as described above. In this case, however, the presence of specifically bound HC-V fragments was detected by incubation with monoclonal anti-myc antibodies, followed by incubation with poly-clonal rabbit-anti-mouse conjugate with alkaline phosphatase. In this way a number of anti-hCG and anti-RR6 HC-V fragments were isolated, which are:

anti-RR6:

R9 pUR4640 (see Figure 1) (SEQ. ID NO. 21-22)

anti-hCG (alpha unit):

H14 pUR4601 (see Figure 2) (SEQ. ID NO. 23-24)

c) Production of llama HC-V biheads by *S. cerevisiae*

i) Construction of episomal expression plasmids encoding anti-hCG/anti-RR6 bispecific biheads

In the anti-hCG HC-V fragment H14 (anti-alpha-subunit), the *Pst*I site was removed and a *Xho*I site was introduced via PCR, using the primers:

MPG158WB

*Xho*I
5'-GAATTAAGCGGCCGCCCAGGTGAAACTGCTCGAGTCWGGGGGA-3' (SEQ. ID NO. 25)

and

MPG159WB

*Bst*EII
3'-CCCTGGGTCCAGTGGCAGAGGAGTGGCAGAGGAGTCTTGTTT-5' (SEQ. ID NO. 26)

In this way the sequence:

*Pst*I
CAG GTC CAG CTG CAG GAG TCT GGG (SEQ. ID NO. 27)
Q V Q L Q E S G

became

XhoI
CAG GTG AAA CTG CTC GAG TCW GGG (SEQ. ID NO. 28)
Q V K L L E S G

Upon digesting the PCR fragments with *Xho*I and *Bst*EII, the about 330bp fragments were purified via agarose gel electrophoresis and isolation from the gel. The fragments were cloned into pUR4421 (see Example 1 in WO 94/25591) which was digested with the same enzymes, resulting in pJS2 (H14). Subsequently, the about 420bp *Eag*I -*Hin*dIII fragment of pJS2 was isolated and ligated in the about 6.6kb *Eag*I- *Hin*dIII vector fragment of the pSY1 plasmid of which the *Pst*I and *Bst*EII sites were removed as described above. The resulting plasmid pJS7, was digested with *Bst*EII and *Hin*dIII, after which the purified vector fragment was religated in the presence of a synthetic linker having the following sequence:

(SEQ. ID NO. 29-31)

```
BstEII                    PstI                    HindIII
 <-            MPG 160 WB (49)                 ->
GGTCACCGTCTCCTCACAGGTGCAGCTGCAGGAGTCACTGTAATGACTTAAGCTT
---------+---------+---------+---------+---------+----- 55
CCAGTGGCAGAGGAGTGTCCACGTCGACGTCCTCAGTGACATTACTGAATTCGAA
     <-          MPG 161 WB (48)                    ->
 V  T  V  S  S  Q  V  Q  L  Q  E  S  L  *  *  L  K  L
```

resulting in plasmid pJS9. Finally, the plasmid was digested with *Pst*I and *Hin*dIII, after which the purified vector fragments of about 7.0kb were ligated with the *Pst*I -*Hin*dIII fragments of about 350bp of pUR4638 and pUR4640, encoding an anti-RR6 HC-V fragment denoted R9 followed by the myc-tail. The resulting *S. cerevisiae* episomal expression plasmid pUR4619 encodes a anti-hCG-anti-RR6 bispecific bihead preceded by the SUC2 signal sequence and followed by the myc-tail.

pUR4619: SUC2 - H14 - R9 - myc

(see Figures 3-4/SEQ. ID NO. 32-33)

Upon digesting these plasmids with *Xho*I and partially with *Bst*EII, *Xho*I-*Bst*EII fragments of about 0.7kb can be isolated and subsequently cloned into the vector fragment of pUR4547 (digested with the same enzymes). In this way biheads can be obtained without the myc tail.

It will be appreciated that expression vectors can be constructed in which different promoter systems, e.g. the constitutive GAPDH promoter or different signal sequences, e.g. the mating factor prepro sequence are used.

ii) Production of the HC-V biheads

After introducing the expression plasmid pUR4619 into *S. cerevisiae* via electroporation, transformants were selected

from minimal medium agar plates as described in part b(ii) above. For the production of biheads, the transformants were grown overnight in selective minimal medium and subsequently diluted ten times in YPGal medium. After 24 and 48 hours of growth, samples were taken for Western blot analysis. For the immuno detection of the produced biheads via Western blot analysis, monoclonal anti-myc antibodies were used, followed by incubation with poly-clonal rabbit-anti-mouse conjugate with alkaline phosphatase.

d) Anti-hCG/anti-RR6 bispecific biheads containing a linker peptide

i) Construction of S. cerevisiae episomal expression plasmids encoding anti-hCG/anti-RR6 bispecific biheads containing a linker peptide

Between the H14 and the R9 encoding DNA fragments synthetic linkers were introduced encoding different linker peptides. To this end the about 50 bp long *Bst*EII-*Hin*dIII fragment of pJS7 (see Example 1 c(i) above) was replaced by an about 50 bp long *Bst*EII-*Hin*dIII fragment having the following sequence:

MVaJA

*Bst*EII *Xba*I *Dra*III *Pst*I *Hin*dIII

5' **GTCACC**GTC**TCTAGA**TGGC**CACCAGGTG**CAG**CTGCAG**GAGTCAACTT**A** 3'

(SEQ. ID NO. 34)

MVbJA

3' G**CAGAGATCT**ACCG**GTGGTCCAC**GTC**GAGCTC**CTCAGTTGAA**TTCGA** 5'

(SEQ. ID NO. 35)

This resulted in pSJ7a. In this plasmid the about 20 bp *Pst*I-*Hin*dIII fragment was replaced with the about 370 bp *Pst*I-*Hin*dIII fragment encoding the anti-RR6 HC-V fragment R9 and/with the myc-tail of pUR4640 (see Example 1 c(i)) and resulting in pSJ7b.

Upon digesting plasmid pSJ7b with *Xba*I and *Dra*III the about 7 kb vector fragment was ligated with five synthetic oligo nucleotide linker fragments presented below:

MV01JA 5′ CTAGTGGTACTTCCGGTTCCCAG 3′ (SEQ. ID NO. 36)

MV02JA 3′ ACCATGAAGGCCAAGG 5′ (SEQ. ID NO. 37)

S **G T S G S** Q

MV03JA 5′ CTAGTTCTTCATCTGCTTCTGCCTCTTCAGCCCAG 3′ (SEQ. ID NO. 38)

MV04JA 3′ AAGAAGTAGACGAAGACGGAGAAGTCGG 5′ (SEQ ID NO. 39)

S **S S S A S A S S A** Q

MV05JA 5′CTAGTGGTTCTCCAGGTTCACCAGGTCAG 3′ (SEQ. ID NO. 40)

MV06JA 3′ ACCAAGAGGTCCAAGTGGTCCA 5′ (SEQ. ID NO. 41)

S **G S P G S P G** Q

MV07JA 5′ CTAGTGCTACTACAACTGGTTCTTCACCAGGTCCAACTCAG 3′ (SEQ. ID NO. 42)

MV08JA 3′ ACGATGATGTTGACCAAGAAGTGGTCCAGGTTGA 5′ (SEQ. ID NO. 43)

S **A T T T G S S P G P T** Q

MV09JA 5′ CTAGTGCTAATCATTCTGGTAATGCTTCTCAG 3′

(SEQ. ID NO. 44)

MV10JA 3′ ACGATTAGTAAGACCATTACGAAGA 5′ (SEQ. ID NO. 45)

S **A N H S G N A S** Q

The oligonucleotide linker fragments encode the last amino acid of the N-terminal HC-V fragment (S) and the first amino acid of the C-terminal HC-V fragment, intersected by the connecting linker peptide. This resulted in plasmids pUR5330 to 5334, respectively.

After transformation of *S. cerevisiae* with these plasmids, the production levels of the biheads were determined via Western blot analysis and a anti-hCG ELISA using anti-myc mAb for detection of the bound bihead (see Example 1 b(iii). Production levels are presented in Table 2 below:

Table 2

Plasmid	Linker	Production level (mg/l)
pUR4619	None	11
pUR5330	S-**G-T-S-G-S**-Q	36
pUR5331	S-**S-S-S-A-S-A-S-S-A**-Q	49
pUR5332	S-**G-S-P-G-S-P-G**-Q	33
pUR5333	S-**A-T-T-T-G-S-S-P-G-P-T**-Q	56
pUR5334	S-**A-N-H-S-G-N-A-S**-Q	51

The production levels of the biheads in which the two HC-V domains are separated by a linker peptide (consisting of between 5 and 11 amino acids) were found to be 3 to 5 times higher as found for the bihead in which the two HC-V fragments are connected without a peptide linker.

Finally, the bispecificity of the biheads was demonstrated as follows:

PINs coated with hCG were incubated with (diluted) medium samples. Subsequently, the PINs were incubated with a RR6-alkaline phosphatase conjugate, in which the azo-dye RR6 was coupled to the alkaline phosphatase via its reactive triazine group. Finally, the alkaline phosphatase enzyme activity was determined after incubation of the PINs with p-nitro-phenyl phosphate and the optical density was measured at 405nm (see Figure 5).

1.2 Purification of Llama Bi-heads with Various Linkers from *S. cerevisiae* Culture Media

A 5 ml column of recombinant Protein A Fast Flow Sepharose (Amersham Pharmacia Biotech) was equilibrated by washing with 10 column volumes of wash buffer (10 mM potassium phosphate, pH 6), at a flow rate of 2 ml/min. The bi-head fermentation broth was loaded at 2 ml/min in an upwards direction. After loading, the column was washed with wash buffer until the OD_{280} reached the baseline. Elution was carried out with a linear gradient of 0 - 40 mM citric acid pH 2.5 in the reverse direction, collecting 4 ml fractions into tubes containing 400 µl of a neutralising agent (1M Tris.Cl, pH 8.5) in order to minimise the effects of the acid. Peak fractions were checked for purity by running on a 12% SDS-PAGE Ready Gel (Bio-Rad) under standard denaturing conditions. Staining was with GelCode Blue (Pierce & Warriner). The fractions were concentrated using Macrosep centrifugal concentrators (3 kDa molecular weight cut-off, Pall Filtron Corp.) then buffer exchanged into 10mM potassium phosphate, pH 6 using PD-10 columns (Amersham Pharmacia Biotech). The final purity of the sample was determined by carrying out a UV scan from 400 - 220 nm and using the value at 280 nm to determine an

accurate concentration. The samples were then aliquoted into vials, frozen, freeze dried and stored until required.

1.3 Preparation of a Reactive Red 6-Bovine Serum Albumin Conjugate

A solution of Reactive Red 6 (RR6) was made up at 10 mg/ml in phosphate buffered saline (PBS). A solution of bovine serum albumin (BSA) was made up at 10 mg/ml in PBS. 200 µl of the RR6 solution was added to 800 µl of the BSA solution and the resulting solution was mixed in an end over end rotary mixer for 2 hours at room temperature. RR6 that had conugated to BSA was separated from free RR6 by addition of the reaction mixture (1 ml) to a PD10 column (Pharmacia) previously washed with 10 ml of PBS containing 0.1% sodium azide (PBSA). The column was then eluted by addition of PBSA (5 ml) and 1 ml aliquots were collected. The RR6-BSA conjugate eluted in fractions 4 and 5. These were pooled and the concentration of protein was determined using a BCA protein test and the concentration adjusted to 2 mg/ml with PBSA.

1.4 Adsorption of Latex with Reactive Red 6-Bovine Serum Albumin Conjugate

Duke blue latex was adsorbed with the RR6-BSA conjugate as follows:

To 950 µl of 10 mM borate buffer, 0.01 % merthiolate, pH 8.5 (buffer B) a 50 µl aliqot of Duke blue latex (10 % solids) was added and mixed by inverting. The diluted latex was centrifuged at 8000 g for 10 minutes at room temperature, the supernatant removed and the pellet vortexed briefly. The pellet was resuspended in 900 µl of buffer B and to this 100 µl of the previously prepared RR6-BSA conjugate was added. The latex solution was sonicated for 10 s using a sonic probe. The

solution containing the latex was mixed for 2 h at room temperature and then centrifuged (8000 g, 10 min at room temperature). The latex pellet was washed by resuspending in 1 ml of buffer B and centrifuged once more (8000 g, 10 min at room temperature). The pellet was then resuspended in 1 ml buffer B ready for use.

1.5 Analysis of Llama Bi-head Self Assembling on Reactive Red 6-Bovine Serum Albumin Adsorbed Latex

The llama bi-heads were tested by self assembling onto RR6-BSA adsorbed latex and detection of hCG in a rapid assay technology (RAT) format. This was performed by mixing the llama bi-head (5 µl of a 0.1 mg/ml solution) with RR6-BSA adsorbed latex (5 µl of 0.1 % solids) in 10 µl of PBSA to which hCG (5 µl of various concentrations) was added. The resulting solution was added to the bottom of a nitrocellulose strip (6 mm wide x 30 mm long) on which a monoclonal antibody recognising hCG had been adsorbed by plotting in a line (2.5 mg/ml) mid way up the strip. The latex-bi-head-hCG solution was allowed to flow up the nitrocellulose strip by capillary action and the strip was then washed by applying PBSA (25 µl) to the bottom of the strip. The amount of latex, captured at the plotted antibody line on the nitrocellulose strip, was quantified by measuring the absorbance through the strip.

Figure 6 shows that the llama bi-heads with linkers 3, 4 and 5 gave the highest response in RAT assays. These linkers are structurally more ordered than the comparative examples, flexible linkers 2 and 6 and result in more hCG and more latex captured in the assay. The more ordered linkers promote the correct orientation of the binding domains to achieve more optimal binding than when no linker is used. Linker 3 is derived from CWP1 and Linker 5 from CBH1P.

Synthetic linkers with some order (linker 4 containing 2 proline residues) can offer increased sensitivity in assays than those with little order (linker 2). Figure 7 shows that the bi-head with linker 4 can detect lower amounts (50 mIU/ml) of hCG than the bi-head with linker 2 and, hence, give a more sensitive assay for hCG.

CLAIMS

1. Use of a polypeptide group, the amino acid sequence of which group confers restricted conformational flexibility, as a linking group to link binding units in a multivalent binding protein.

2. Use according to claim 1 wherein the polypeptide linking group comprises from 4 to 30 amino acid residues.

3. Use according to claim 1 or 2 wherein the linking group comprises one or more proline residues.

4. Use according to claim 1 or 2 wherein the linking group comprises an amino acid sequence selected from:

S-S-S-A-S-A-S-S-A,
G-S-P-G-S-P-G, or
A-T-T-T-G-S-S-P-G-P-T.

5. A multivalent binding protein comprising a plurality of binding units linked by means of intervening polypeptide linker groups, the amino acid sequence of which linker group confers restricted conformational flexibility.

6. A protein according to claim 5 wherein the binding units comprise heavy chain variable domains derived from an immunoglobulin naturally devoid of light chains.

7. A protein according to claim 5 or claim 6 wherein the antigen binding units comprise heavy chain variable domains derived from a Camelid immunoglobulin.

8. A protein according to any one of claims 5 to 7 comprising a bivalent antigen binding protein.

9. A protein according to any one of claims 5 to 8 wherein the linker group comprises from 4 to 30 amino acid residues.

10. A protein according to any one of claims 5 to 9 wherein the linker group comprises one or more proline residues.

11. A protein according to any one of claims 5 to 9 wherein the linker group comprises an amino acid sequence selected from:

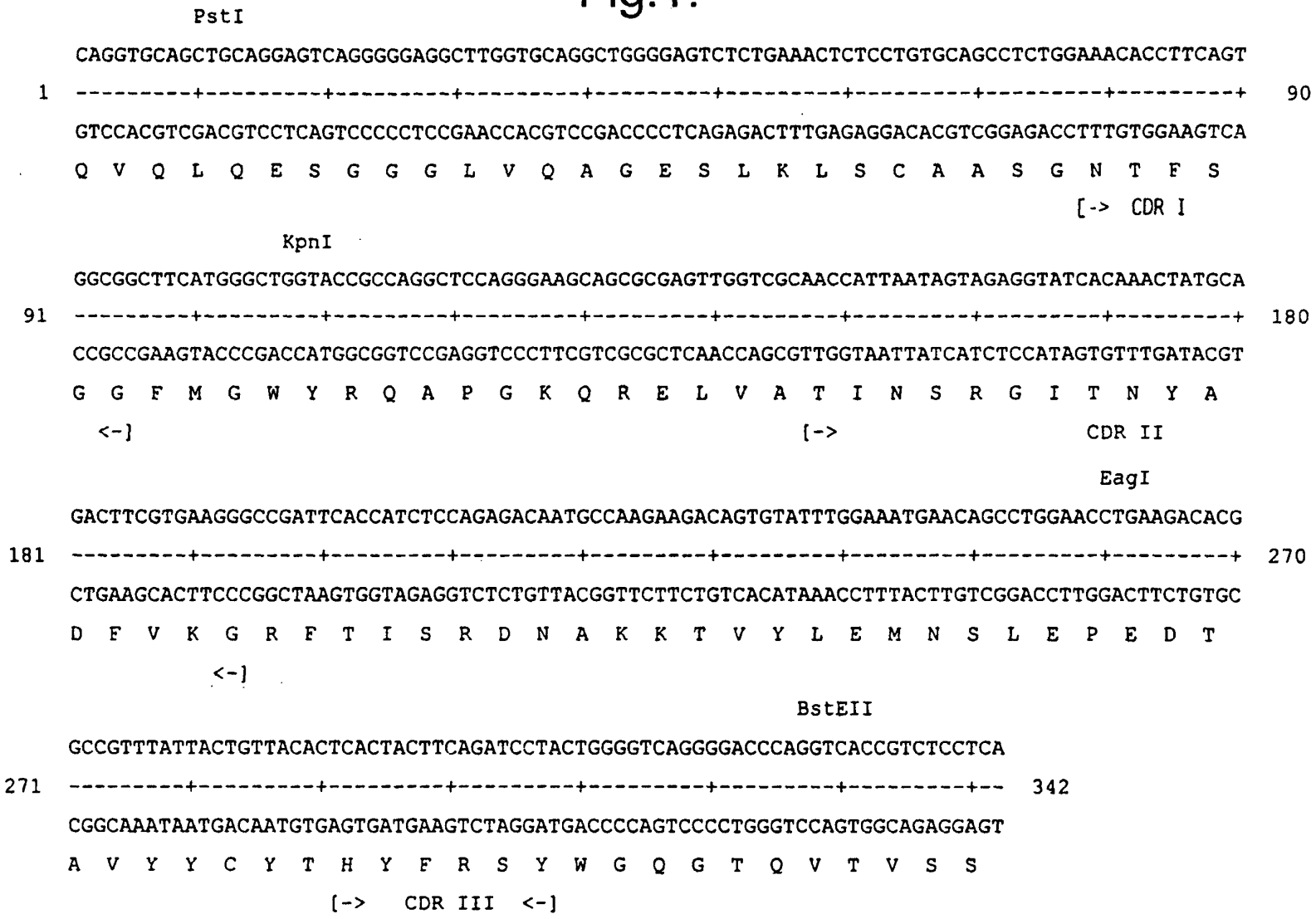
S-S-S-A-S-A-S-S-A,
G-S-P-G-S-P-G, or
A-T-T-T-G-S-S-P-G-P-T.

12. Nucleotide sequences encoding for a multivalent binding protein of any one of claims 5 to 11.

13. An expression vector comprising a nucleotide sequence according to claim 12.

14. A host cell transformed with a vector according to claim 13.

Fig.1.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[-> CDR I
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<-]
[->
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Fig.2.

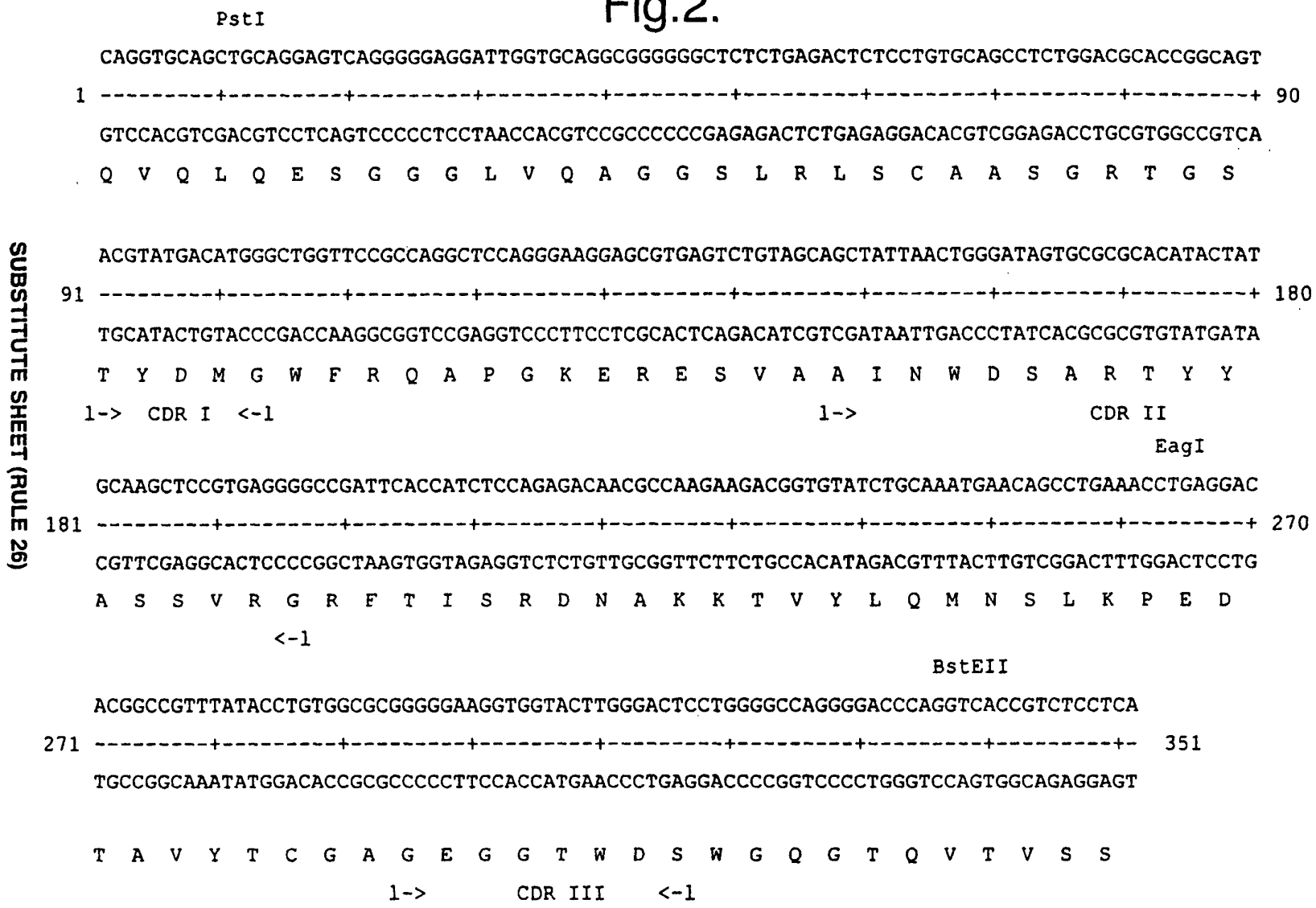
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Fig.3.

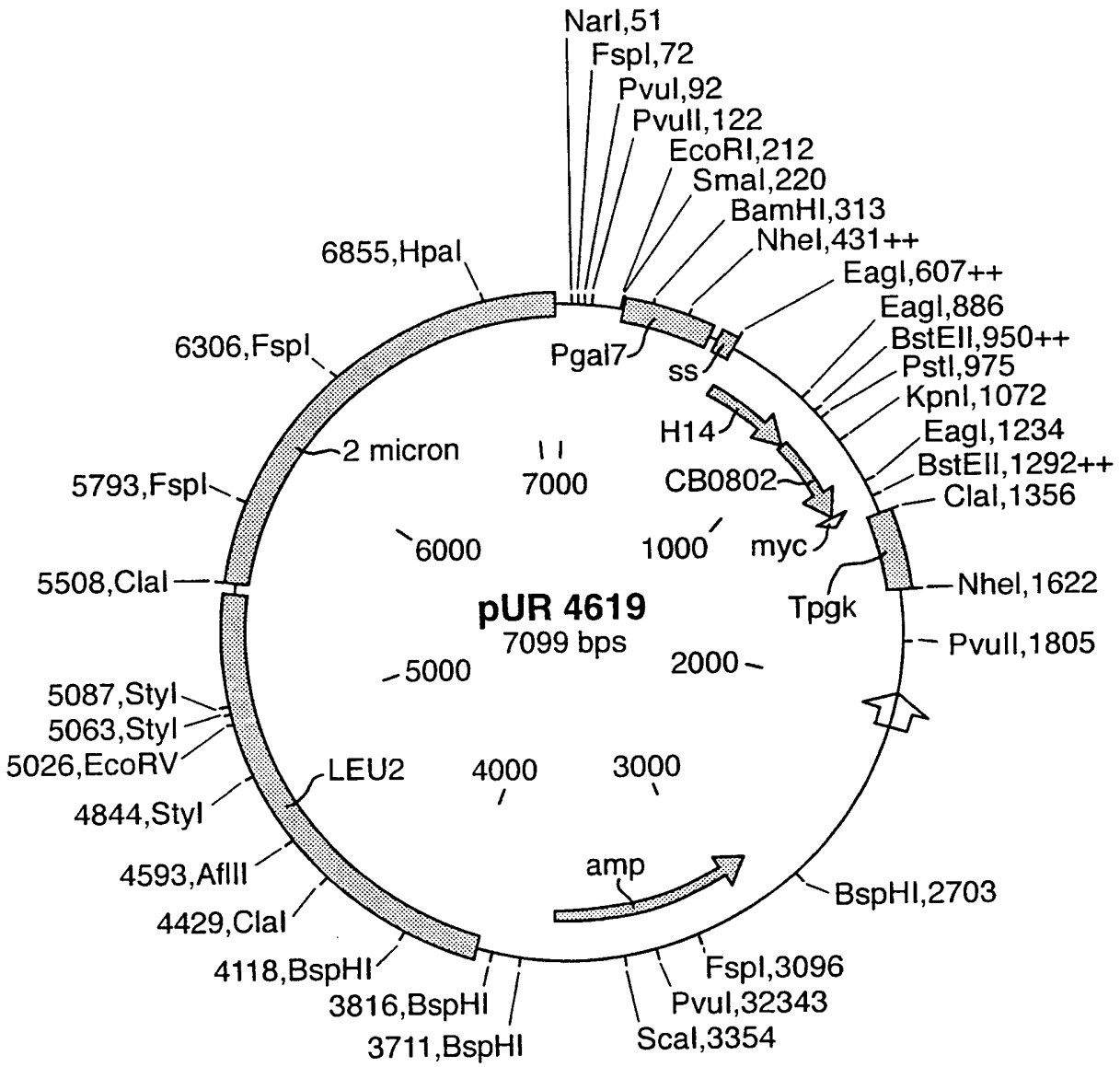
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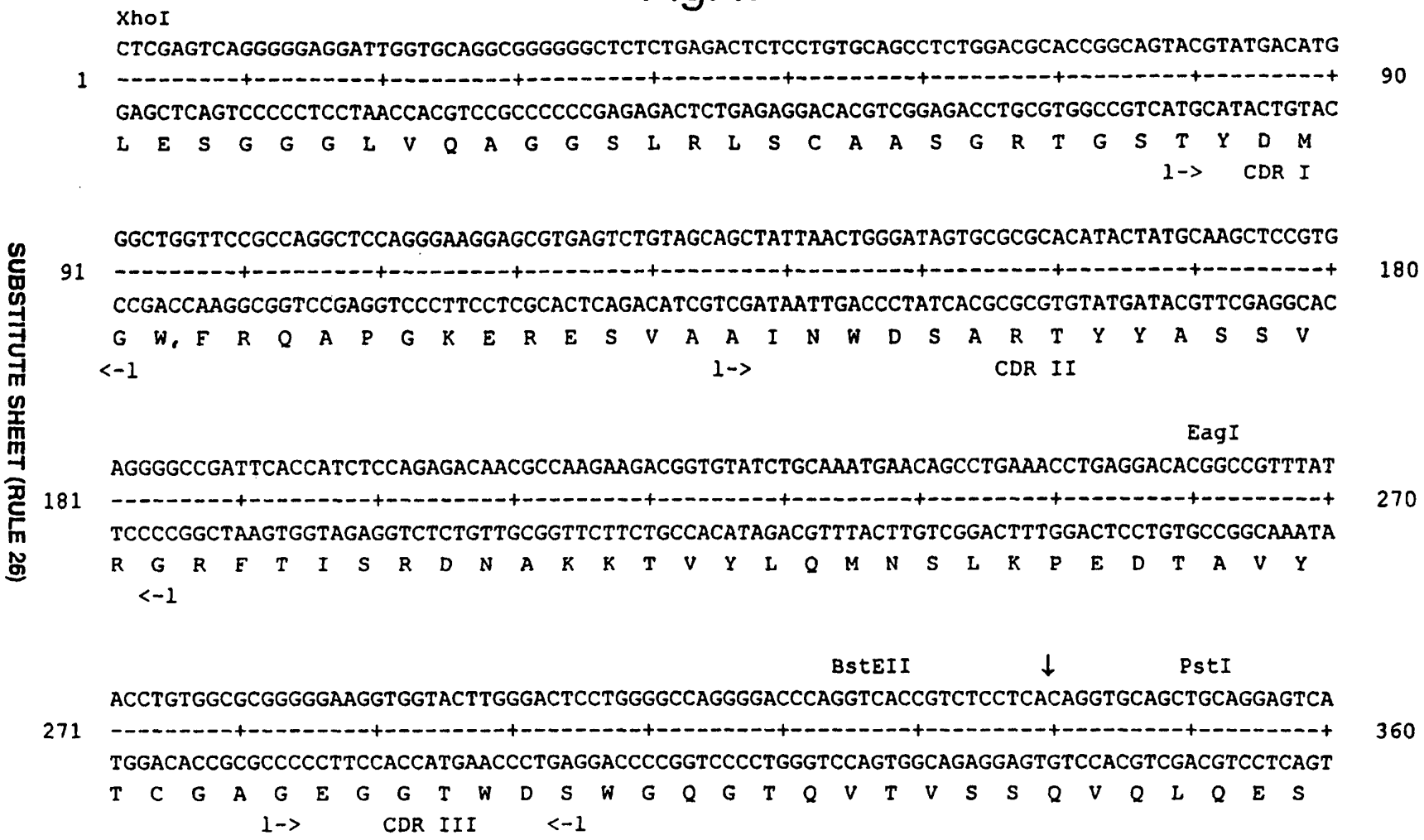
Fig.4.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L E S G G G L V Q A G G S L R L S C A A S G R T G S T Y D M
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91
180
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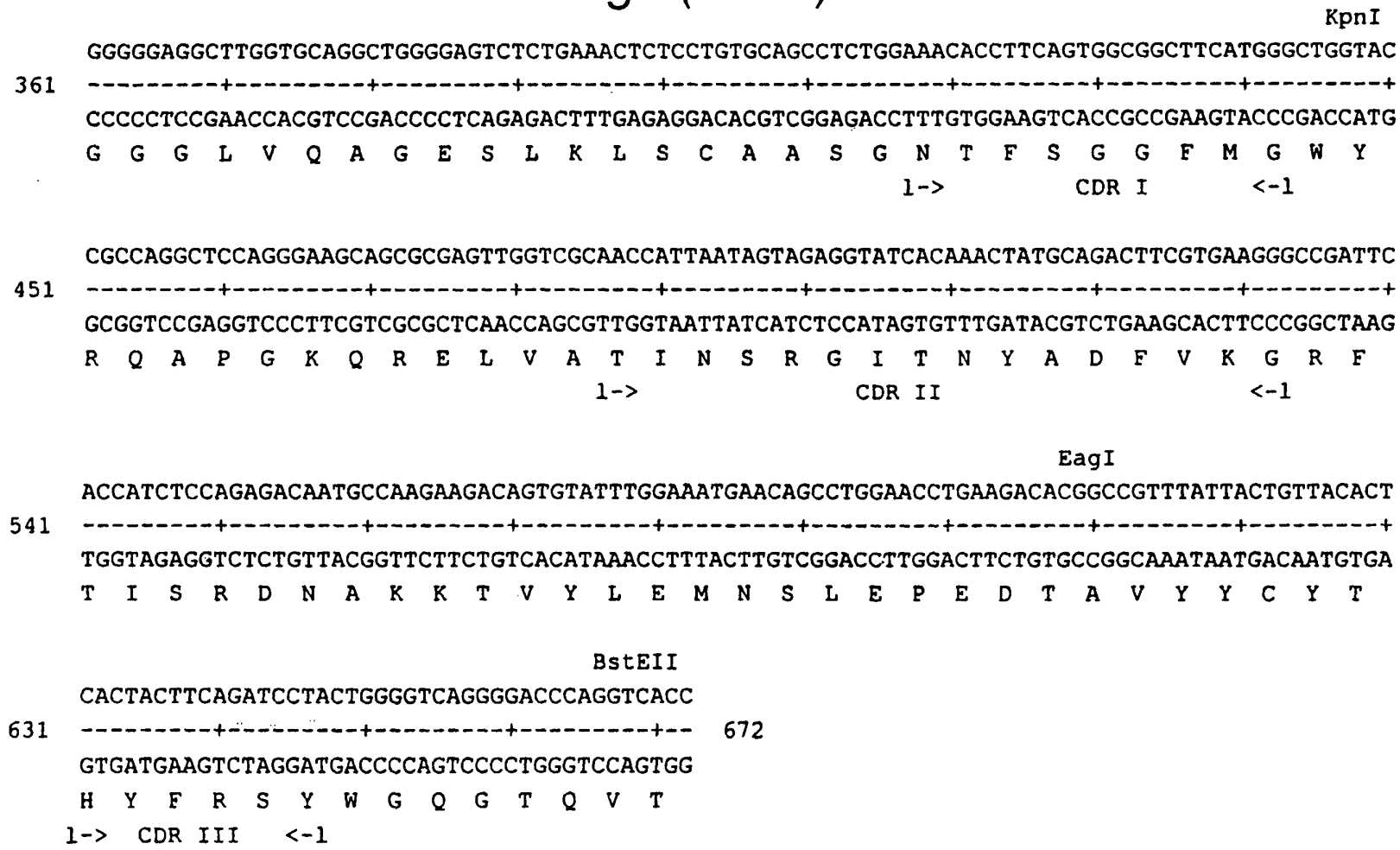
Fig.4(Cont.)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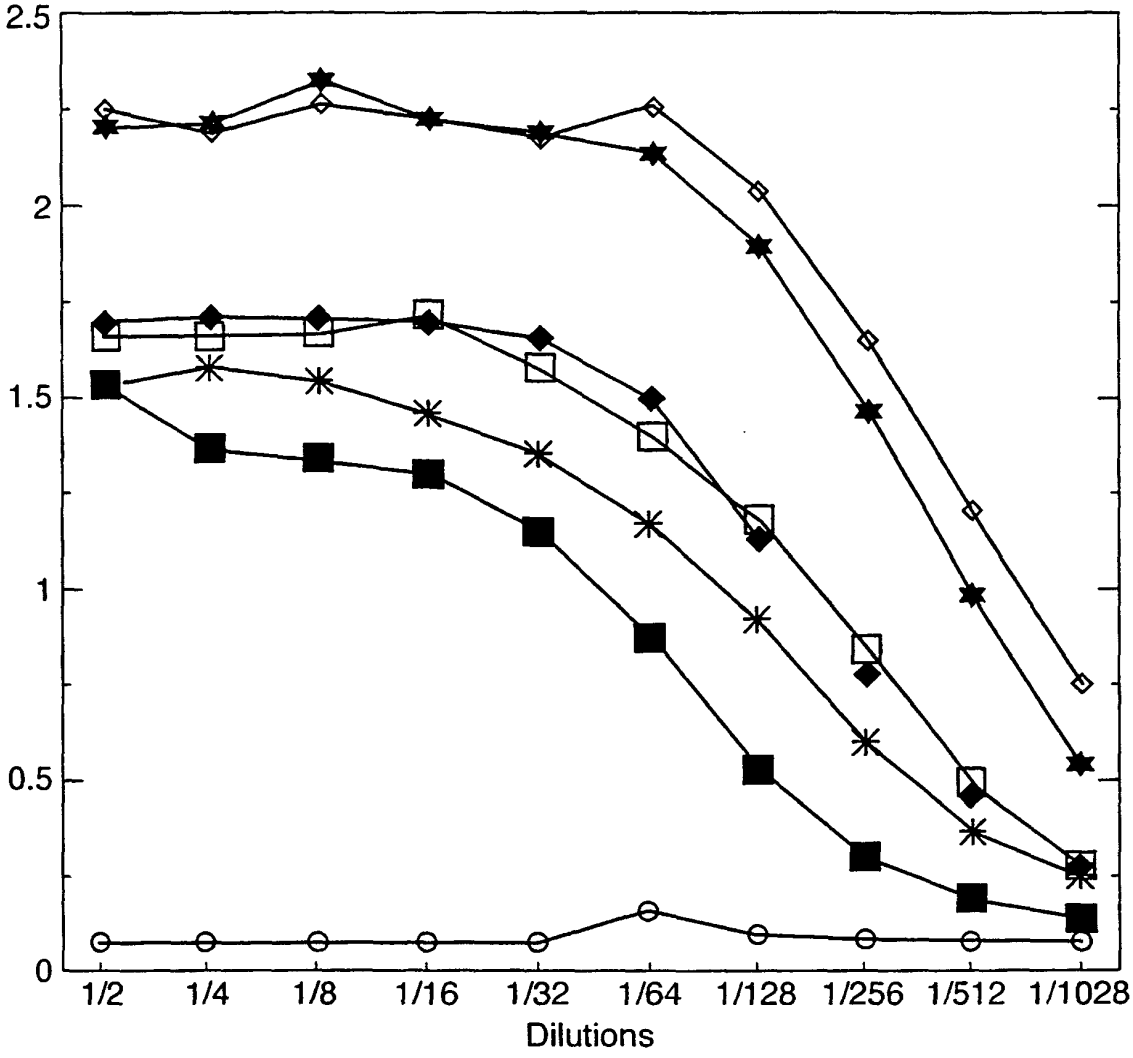
CGCCAGGCTCCAGGGAAGCAGCGCGAGTTGGTCGCAACCATTAATAGTAGAGGTATCACAAACTATGCAGACTTCGTGAAGGGCCGATTC
451
540
GCGGTCCGAGGTCCCTTCGTCGCGCTCAACCAGCGTTGGTAATTATCATCTCCATAGTGTTTGATACGTCTGAAGCACTTCCCGGCTAAG
R Q A P G K Q R E L V A T I N S R G I T N Y A D F V K G R F
1-> CDR II <-1
EagI
ACCATCTCCAGAGACAATGCCAAGAAGACAGTGTATTTGGAAATGAACAGCCTGGAACCTGAAGACACGGCCGTTTATTACTGTTACACT
541
630
TGGTAGAGGTCTCTGTTACGGTTCTTCTGTCACATAAACCTTTACTTGTCGGACCTTGGACTTCTGTGCCGGCAAATAATGACAATGTGA
T I S R D N A K K T V Y L E M N S L E P E D T A V Y Y C Y T
BstEII
CACTACTTCAGATCCTACTGGGGTCAGGGGACCCAGGTCACC
631
672
GTGATGAAGTCTAGGATGACCCCAGTCCCCTGGGTCCAGTGG
H Y F R S Y W G Q G T Q V T
1-> CDR III <-1

Fig.5.


2.5
2
1.5
1
0.5
0
1/2
1/4
1/8
1/16
1/32
1/64
1/128
1/256
1/512
1/1028
Dilutions
pUR4619
pUR5330
pUR5331
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pUR5333
pUR5334
blanc

Fig.6.

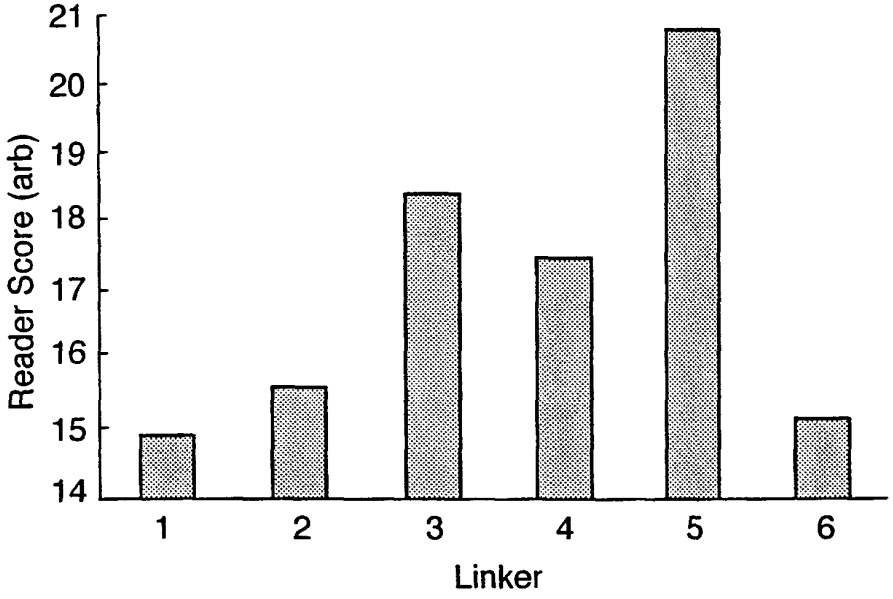
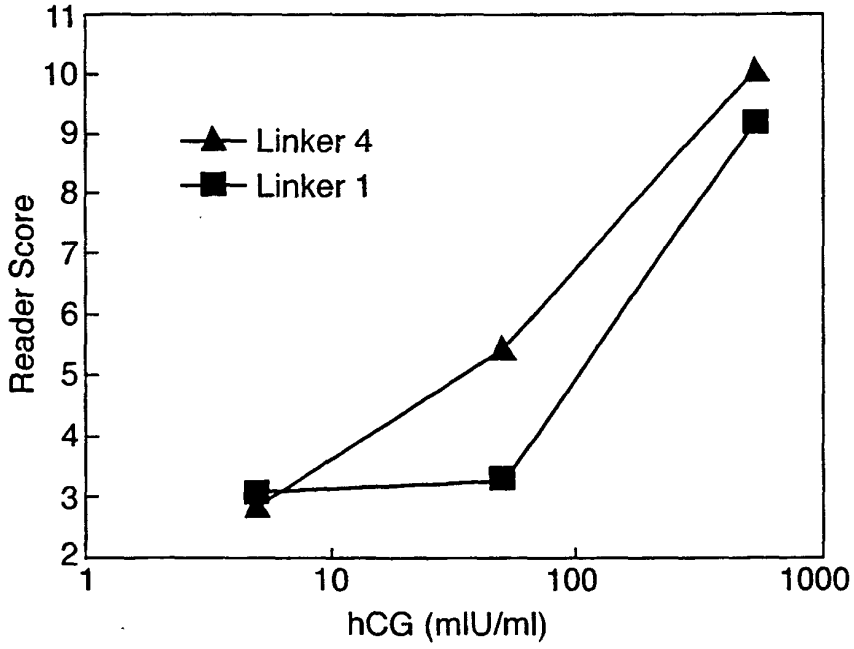

Reader Score (arb)
21
20
19
18
17
16
15
14
1
2
3
4
5
6
Linker

Fig.7.


Reader Score
11
10
9
8
7
6
5
4
3
2
Linker 4
Linker 1
1
10
100
1000
hCG (mIU/ml)

SEQUENCE LISTING

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UNILEVER N.V

<120> ANTIGEN BINDING PROTEINS

<130> T3077

<140>
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<160> 45

<170> PatentIn Ver. 2.1

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<210> 2
<211> 9
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<210> 3
<211> 7
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<400> 3
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1 5

<210> 4
<211> 11
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<213> Artificial Sequence

<220>
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<210> 5
<211> 8
<212> PRT
<213> Artificial Sequence

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<400> 5
Ala Asn His Ser Gly Asn Ala Ser
1 5

<210> 6
<211> 22
<212> DNA
<213> Artificial Sequence

<220>
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<400> 6
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<210> 7
<211> 53
<212> DNA
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<223> Description of Artificial Sequence:PRIMER

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aacagttaag cttccgcttg cggccgcgga gctggggtct tcgctgtggt gcg 53

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<212> DNA
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<223> Description of Artificial Sequence:PRIMER

<400> 8
aacagttaag cttccgcttg cggccgctgg ttgtggtttt ggtgtcttgg gtt 53

<210> 9
<211> 28
<212> DNA
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<400> 9
gggaattcca ataggtggtt agcaatcg 28

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<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:PRIMER

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gaccaacgtg gtcgcctggc aaaacg 26

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<400> 11
cgttttgcca ggcgaccacg ttggtc 26

<210> 12
<211> 30
<212> DNA
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<223> Description of Artificial Sequence:PRIMER

<400> 12
ccccaagctt acatggtctt aagttggcgt 30

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<222> (147)..(155)

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Ala His His Thr Asn Lys Gln Asn Lys Met Met Leu Leu Gln Ala
1 5 10 15

ttc ctt ttc ctt ttg gct ggt ttt gca gcc aaa ata tct gcg cag gtg 95
Phe Leu Phe Leu Leu Ala Gly Phe Ala Ala Lys Ile Ser Ala Gln Val
20 25 30

cag ctg cag gag tca taatga ggg acc cag gtc acc gtc tcc tca taatga 146
Gln Leu Gln Glu Ser Gly Thr Gln Val Thr Val Ser Ser
35 40

ctt aag ctt 155
Leu Lys Leu
45

<210> 14
<211> 36
<212> PRT
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<223> Description of Artificial Sequence:PLASMID CONSTRUCT

<400> 14
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1 5 10 15

Leu Phe Leu Leu Ala Gly Phe Ala Ala Lys Ile Ser Ala Gln Val Gln
20 25 30

Leu Gln Glu Ser
35

<210> 15
<211> 8
<212> PRT
<213> Artificial Sequence
<223> Description of Artificial Sequence:PLASMID CONSTRUCT

<400> 15
Gly Thr Gln Val Thr Val Ser Ser
1 5

<210> 16
<211> 3
<212> PRT
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<223> Description of Artificial Sequence:PLASMID CONSTRUCT

<400> 16
Leu Lys Leu
1

<210> 17
<211> 188
<212> DNA
<213> Artificial Sequence

<220>
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<220>
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Ala His His Thr Asn Lys Gln Asn Lys Met Met Leu Leu Gln Ala
1 5 10 15

ttc ctt ttc ctt ttg gct ggt ttt gca gcc aaa ata tct gcg cag gtg 95
Phe Leu Phe Leu Leu Ala Gly Phe Ala Ala Lys Ile Ser Ala Gln Val
20 25 30

cag ctg cag gag tca taatga ggg acc cag gtc acc gtc tcc tca gaa 143
Gln Leu Gln Glu Ser Gly Thr Gln Val Thr Val Ser Ser Glu
35 40 45

caa aaa ctc atc tca gaa gag gat ctg aat taatga ctt aag ctt 188
Gln Lys Leu Ile Ser Glu Glu Asp Leu Xaa Xaa Lys Leu
50 55

<210> 18
<211> 36
<212> PRT
<213> Artificial Sequence
<223> Description of Artificial Sequence:PLASMID
CONSTRUCT

<400> 18
Ala His His Thr Asn Lys Gln Asn Lys Met Met Leu Leu Gln Ala Phe
1 5 10 15

Leu Phe Leu Leu Ala Gly Phe Ala Ala Lys Ile Ser Ala Gln Val Gln
20 25 30

Leu Gln Glu Ser
35

<210> 19
<211> 19
<212> PRT
<213> Artificial Sequence
<223> Description of Artificial Sequence:PLASMID
CONSTRUCT

<400> 19
Gly Thr Gln Val Thr Val Ser Ser Glu Gln Lys Leu Ile Ser Glu Glu
1 5 10 15

Asp Leu Xaa

<210> 20
<211> 3
<212> PRT
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<223> Description of Artificial Sequence:PLASMID
CONSTRUCT

<400> 20
Xaa Lys Leu
1

<210> 21
<211> 342
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:PLASMID CONSTRUCT

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Gln Val Gln Leu Gln Glu Ser Gly Gly Gly Leu Val Gln Ala Gly Glu
1 5 10 15

tct ctg aaa ctc tcc tgt gca gcc tct gga aac acc ttc agt ggc ggc 96
Ser Leu Lys Leu Ser Cys Ala Ala Ser Gly Asn Thr Phe Ser Gly Gly
20 25 30

ttc atg ggc tgg tac cgc cag gct cca ggg aag cag cgc gag ttg gtc 144
Phe Met Gly Trp Tyr Arg Gln Ala Pro Gly Lys Gln Arg Glu Leu Val
35 40 45

gca acc att aat agt aga ggt atc aca aac tat gca gac ttc gtg aag 192
Ala Thr Ile Asn Ser Arg Gly Ile Thr Asn Tyr Ala Asp Phe Val Lys
50 55 60

ggc cga ttc acc atc tcc aga gac aat gcc aag aag aca gtg tat ttg 240
Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Lys Thr Val Tyr Leu
65 70 75 80

gaa atg aac agc ctg gaa cct gaa gac acg gcc gtt tat tac tgt tac 288
Glu Met Asn Ser Leu Glu Pro Glu Asp Thr Ala Val Tyr Tyr Cys Tyr
85 90 95

act cac tac ttc aga tcc tac tgg ggt cag ggg acc cag gtc acc gtc 336
Thr His Tyr Phe Arg Ser Tyr Trp Gly Gln Gly Thr Gln Val Thr Val
100 105 110

tcc tca 342
Ser Ser

<210> 22
<211> 114
<212> PRT
<213> Artificial Sequence
<223> Description of Artificial Sequence:PLASMID CONSTRUCT

<400> 22
Gln Val Gln Leu Gln Glu Ser Gly Gly Gly Leu Val Gln Ala Gly Glu
1 5 10 15

Ser Leu Lys Leu Ser Cys Ala Ala Ser Gly Asn Thr Phe Ser Gly Gly
20 25 30

Phe Met Gly Trp Tyr Arg Gln Ala Pro Gly Lys Gln Arg Glu Leu Val
35 40 45

Ala Thr Ile Asn Ser Arg Gly Ile Thr Asn Tyr Ala Asp Phe Val Lys
50 55 60

Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Lys Thr Val Tyr Leu
65 70 75 80

Glu Met Asn Ser Leu Glu Pro Glu Asp Thr Ala Val Tyr Tyr Cys Tyr
85 90 95

Thr His Tyr Phe Arg Ser Tyr Trp Gly Gln Gly Thr Gln Val Thr Val
100 105 110

Ser Ser

<210> 23
<211> 351
<212> DNA
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<220>
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<220>
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<222> (1)..(351)

<400> 23

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Gln Val Gln Leu Gln Glu Ser Gly Gly Gly Leu Val Gln Ala Gly Gly
 1               5                  10                  15

tct ctg aga ctc tcc tgt gca gcc tct gga cgc acc ggc agt acg tat   96
Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Arg Thr Gly Ser Thr Tyr
            20                  25                  30

gac atg ggc tgg ttc cgc cag gct cca ggg aag gag cgt gag tct gta   144
Asp Met Gly Trp Phe Arg Gln Ala Pro Gly Lys Glu Arg Glu Ser Val
        35                  40                  45

gca gct att aac tgg gat agt gcg cgc aca tac tat gca agc tcc gtg   192
Ala Ala Ile Asn Trp Asp Ser Ala Arg Thr Tyr Tyr Ala Ser Ser Val
    50                  55                  60

agg ggc cga ttc acc atc tcc aga gac aac gcc aag aag acg gtg tat   240
Arg Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Lys Thr Val Tyr
65                  70                  75                  80

ctg caa atg aac agc ctg aaa cct gag gac acg gcc gtt tat acc tgt   288
Leu Gln Met Asn Ser Leu Lys Pro Glu Asp Thr Ala Val Tyr Thr Cys
                85                  90                  95

ggc gcg ggg gaa ggt ggt act tgg gac tcc tgg ggc cag ggg acc cag   336
Gly Ala Gly Glu Gly Gly Thr Trp Asp Ser Trp Gly Gln Gly Thr Gln
            100                 105                 110

gtc acc gtc tcc tca                                              351
Val Thr Val Ser Ser
        115
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<211> 117
<212> PRT
<213> Artificial Sequence
<223> Description of Artificial Sequence:PLASMID CONSTRUCT

<400> 24

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Ser	Leu	Arg	Leu	Ser	Cys	Ala	Ala	Ser	Gly	Arg	Thr	Gly	Ser	Thr	Tyr
			20					25					30		
Asp	Met	Gly	Trp	Phe	Arg	Gln	Ala	Pro	Gly	Lys	Glu	Arg	Glu	Ser	Val
		35					40					45			
Ala	Ala	Ile	Asn	Trp	Asp	Ser	Ala	Arg	Thr	Tyr	Tyr	Ala	Ser	Ser	Val
	50					55					60				
Arg	Gly	Arg	Phe	Thr	Ile	Ser	Arg	Asp	Asn	Ala	Lys	Lys	Thr	Val	Tyr
65					70					75					80
Leu	Gln	Met	Asn	Ser	Leu	Lys	Pro	Glu	Asp	Thr	Ala	Val	Tyr	Thr	Cys
				85					90					95	
Gly	Ala	Gly	Glu	Gly	Gly	Thr	Trp	Asp	Ser	Trp	Gly	Gln	Gly	Thr	Gln
			100					105					110		
Val	Thr	Val	Ser	Ser											
		115													

<210> 25
<211> 43
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:PRIMER

<400> 25
gaattaagcg gccgcccagg tgaaactgct cgagtcwggg gga 43

<210> 26
<211> 42
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:PRIMER

<400> 26
ccctgggtcc agtggcagag gagtggcaga ggagtcttgt tt 42

<210> 27
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:PRIMER

<400> 27
caggtccagc tgcaggagtc tggg 24

<210> 28
<211> 24
<212> DNA
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<220>
<223> Description of Artificial Sequence:PRIMER

<400> 28
caggtgaaac tgctcgagtc wggg 24

<210> 29
<211> 55
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:LINKER;DOUBLE STRANDED

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g gtc acc gtc tcc tca cag gtg cag ctg cag gag tca ctg taatga ctt 49
Val Thr Val Ser Ser Gln Val Gln Leu Gln Glu Ser Xaa Xaa
1 5 10

aag ctt 55
Lys Leu
15

<210> 30
<211> 13
<212> PRT
<213> Artificial Sequence
<223> Description of Artificial Sequence:LINKER;DOUBLE STRANDED

<400> 30
Val Thr Val Ser Ser Gln Val Gln Leu Gln Glu Ser Xaa
1 5 10

<210> 31
<211> 3
<212> PRT
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<400> 31
Xaa Lys Leu

1

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 1               5                  10                  15

tcc tgt gca gcc tct gga cgc acc ggc agt acg tat gac atg ggc tgg   96
Ser Cys Ala Ala Ser Gly Arg Thr Gly Ser Thr Tyr Asp Met Gly Trp
            20                  25                  30

ttc cgc cag gct cca ggg aag gag cgt gag tct gta gca gct att aac   144
Phe Arg Gln Ala Pro Gly Lys Glu Arg Glu Ser Val Ala Ala Ile Asn
        35                  40                  45

tgg gat agt gcg cgc aca tac tat gca agc tcc gtg agg ggc cga ttc   192
Trp Asp Ser Ala Arg Thr Tyr Tyr Ala Ser Ser Val Arg Gly Arg Phe
    50                  55                  60

acc atc tcc aga gac aac gcc aag aag acg gtg tat ctg caa atg aac   240
Thr Ile Ser Arg Asp Asn Ala Lys Lys Thr Val Tyr Leu Gln Met Asn
65                  70                  75                  80

agc ctg aaa cct gag gac acg gcc gtt tat acc tgt ggc gcg ggg gaa   288
Ser Leu Lys Pro Glu Asp Thr Ala Val Tyr Thr Cys Gly Ala Gly Glu
                85                  90                  95

ggt ggt act tgg gac tcc tgg ggc cag ggg acc cag gtc acc gtc tcc   336
Gly Gly Thr Trp Asp Ser Trp Gly Gln Gly Thr Gln Val Thr Val Ser
            100                 105                 110

tca cag gtg cag ctg cag gag tca ggg gga ggc ttg gtg cag gct ggg   384
Ser Gln Val Gln Leu Gln Glu Ser Gly Gly Gly Leu Val Gln Ala Gly
        115                 120                 125

gag tct ctg aaa ctc tcc tgt gca gcc tct gga aac acc ttc agt ggc   432
Glu Ser Leu Lys Leu Ser Cys Ala Ala Ser Gly Asn Thr Phe Ser Gly
    130                 135                 140

ggc ttc atg ggc tgg tac cgc cag gct cca ggg aag cag cgc gag ttg   480
Gly Phe Met Gly Trp Tyr Arg Gln Ala Pro Gly Lys Gln Arg Glu Leu
145                 150                 155                 160

gtc gca acc att aat agt aga ggt atc aca aac tat gca gac ttc gtg   528
Val Ala Thr Ile Asn Ser Arg Gly Ile Thr Asn Tyr Ala Asp Phe Val
                165                 170                 175

aag ggc cga ttc acc atc tcc aga gac aat gcc aag aag aca gtg tat   576
Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Lys Thr Val Tyr
            180                 185                 190
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ttg gaa atg aac agc ctg gaa cct gaa gac acg gcc gtt tat tac tgt   624
Leu Glu Met Asn Ser Leu Glu Pro Glu Asp Thr Ala Val Tyr Tyr Cys
        195                 200                 205

tac act cac tac ttc aga tcc tac tgg ggt cag ggg acc cag gtc acc   672
Tyr Thr His Tyr Phe Arg Ser Tyr Trp Gly Gln Gly Thr Gln Val Thr
    210                 215                 220
```

<210> 33
<211> 224
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<400> 33

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Leu Glu Ser Gly Gly Gly Leu Val Gln Ala Gly Gly Ser Leu Arg Leu
  1               5                  10                  15

Ser Cys Ala Ala Ser Gly Arg Thr Gly Ser Thr Tyr Asp Met Gly Trp
             20                  25                  30

Phe Arg Gln Ala Pro Gly Lys Glu Arg Glu Ser Val Ala Ala Ile Asn
         35                  40                  45

Trp Asp Ser Ala Arg Thr Tyr Tyr Ala Ser Ser Val Arg Gly Arg Phe
     50                  55                  60

Thr Ile Ser Arg Asp Asn Ala Lys Lys Thr Val Tyr Leu Gln Met Asn
 65                  70                  75                  80

Ser Leu Lys Pro Glu Asp Thr Ala Val Tyr Thr Cys Gly Ala Gly Glu
                 85                  90                  95

Gly Gly Thr Trp Asp Ser Trp Gly Gln Gly Thr Gln Val Thr Val Ser
            100                 105                 110

Ser Gln Val Gln Leu Gln Glu Ser Gly Gly Gly Leu Val Gln Ala Gly
        115                 120                 125

Glu Ser Leu Lys Leu Ser Cys Ala Ala Ser Gly Asn Thr Phe Ser Gly
    130                 135                 140

Gly Phe Met Gly Trp Tyr Arg Gln Ala Pro Gly Lys Gln Arg Glu Leu
145                 150                 155                 160

Val Ala Thr Ile Asn Ser Arg Gly Ile Thr Asn Tyr Ala Asp Phe Val
                165                 170                 175

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Lys Thr Val Tyr
            180                 185                 190

Leu Glu Met Asn Ser Leu Glu Pro Glu Asp Thr Ala Val Tyr Tyr Cys
        195                 200                 205

Tyr Thr His Tyr Phe Arg Ser Tyr Trp Gly Gln Gly Thr Gln Val Thr
    210                 215                 220
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<213> Artificial Sequence

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<210> 35
<211> 47
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:SYNTHETIC INSERT

<400> 35
gcagagatct accggtggtc cacgtcgagc tcctcagttg aattcga 47

<210> 36
<211> 23
<212> DNA
<213> Artificial Sequence

<220>
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<400> 36
ctagtggtac ttccggttcc cag 23

<210> 37
<211> 16
<212> DNA
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<220>
<223> Description of Artificial Sequence:LINKER

<400> 37
accatgaagg ccaagg 16

<210> 38
<211> 35
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<223> Description of Artificial Sequence:LINKER

<400> 38
ctagttcttc atctgcttct gcctcttcag cccag 35

<210> 39
<211> 28
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<400> 39
aagaagtaga cgaagacgga gaagtcgg 28

<210> 40
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<223> Description of Artificial Sequence:LINKER

<400> 40
ctagtggttc tccaggttca ccaggtcag 29

<210> 41
<211> 22
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:LINKER

<400> 41
accaagaggt ccaagtggtc ca 22

<210> 42
<211> 41
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:LINKER

<400> 42
ctagtgctac tacaactggt tcttcaccag gtccaactca g 41

<210> 43
<211> 34
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:LINKER

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acgatgatgt tgaccaagaa gtggtccagg ttga 34

<210> 44
<211> 32
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:LINKER

<400> 44
ctagtgctaa tcattctggt aatgcttctc ag 32

<210> 45

<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:LINKER

<400> 45
acgattagta agaccattac gaaga 25